Effects of air pollution on haemostasis and atherosclerosis

Effects of Air Pollution on Haemostasis and Atherosclerosis

Thesis Maastricht University

ISBN 978-90-818447-0-3

Printed by Palme Publications

Coverdesign: Abdulkadir Açıl & Evren KILINÇ

Layout: Evren KILINÇ

Effects of Air Pollution on Haemostasis and Atherosclerosis

Dissertation

To obtain the degree of Doctor at Maastricht University,

on the authority of the Rector Magnificus Prof. Dr. G. P.M. F. Mols,

in accordance with the decision of the Board of Deans,

to be defended in public on Thursday 22nd of December 2011 at 1600 hours

by

Evren KILINÇ

was born on 26th of October 1980, Yeşilyurt/ Malatya, TURKEY

Promotor:

Prof. dr. H. ten Cate

Co-promotor:

Dr. H.M.H. Spronk

Assessment Committee:

Prof. dr. E.A.L. Biessen (Chairman)

Prof. dr. A. Bast

Prof. dr. F.J. van Schooten

Prof. dr. P.H. Reitsma (LUMC, Leiden)

Prof. dr. T. van der Poll (AMC, Amsterdam)

The research described in this thesis was supported by a grant of the Netherlands Heart Foundation (2006B64).

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

Thomas Edison

Contents

CHAPTER 1

GENERAL INTRODUCTION

PART I:
PARTICLES, COAGULATION, AND THROMBOSIS

Based on:

Kilinç, E., Rudež, G., Spronk, H.M.H., Nemmar, A., de Maat, M.P.M., ten Cate, H., Hoylaerts, M.F.

2011. Particles, Coagulation, and Thrombosis in *Cardiovascular Effects of Inhaled Ultrafine and Nano-Sized Particles ed*. Flemming R. Cassee, Nicholas L. Mills, and David E. Newby, pp 405-420. Chicester: John Wiley and Sons Ltd, 2011.

Epidemiological, as well as experimental animal and human volunteer studies, suggest a close link between the risk for myocardial infarction, thrombosis, stroke and death and the inhalation of particulate matter (PM) [1-3]. The mechanisms underlying cardiovascular complications executed by small particles are only partially understood. One potential effect of inhaled particles consists of its impact on the autonomic nervous system, leading to perturbations in heart rhythm [4, 5]. However, additional hypotheses have been formulated to explain the cardiovascular effects of PM. One hypothesis is that PM contribute to the occurrence of pulmonary inflammation, leading to the release of inflammatory mediators, which in turn cause platelet activation and upregulation of coagulation, thus generating or enhancing a protrombotic tendency [6, 7] or worsening vascular dysfunction [8, 9]. Another hypothesis states that some particles can translocate from the lungs into the systemic circulation and thus, directly or indirectly, influence haemostasis or cardiovascular integrity. Evidence for the existence of each mechanism is presented below, based on findings in animal models and association studies.

In this chapter, the effects of PM on coagulation, platelet activation and thrombus formation will be discussed comprehensively.

Haemostasis

Haemostasis is a protective mechanism of the organism in response to vascular injury consisting of two major pathways: the activation and aggregation of thrombocytes or platelets (also called primary haemostasis) and the activation of the coagulation cascade (secondary haemostasis) resulting in an insoluble fibrin clot. The haemostatic system reacts quickly to stop blood loss from a damaged blood vessel wall and seals the wound site with a platelet rich fibrin stabilized clot. In primary haemostasis, circulating platelets bind to subendothelial collagen through their cell surface glycoprotein Ia/IIa receptors to form the primary haemostatic platelet plug. The adhesion of platelets is further stabilized by von Willebrand factor (vWF), which links platelets, glycoproteins and collagen fibrils. The action of a complex cascade of coagulation factors (a group of serine proteases) results in the formation of fibrin strands, which further strengthens the platelet plug [10]. Traditionally, the coagulation cascade has been divided into two distinct pathways: the intrinsic (contact) activation pathway and the extrinsic (tissue factor (TF)) pathway. In the current model of coagulation, TF, a membrane bound glycoprotein, is considered to be the single most relevant trigger of the bloodcoagulation system. The glycoprotein TF is expressed on a number of cells within the

vasculature, including fibroblasts and smooth muscle cells. Circulating blood cells such as monocytes only express this molecule upon induction, for instance by cytokines like Interleukin -6 (IL-6). The exposed TF forms a catalytic complex with factor VIIa (FVIIa) (TF-FVIIa complex) on a phospholipid membrane surface and activates both factor X (FX) and factor IX (FIX) resulting in the activation of prothrombin into thrombin [11]. All these reactions occur on negatively charged phospholipid membranes in the presence of calcium ions. Thrombin generation enhances its own formation through activation of factor XI (FXI) and the cofactors V (FV) and VIII (FVIII). The intrinsic pathway of the coagulation system can also be engaged when plasma coagulation factor XII (FXII), also known as Hageman factor, binds to negatively charged surfaces (note: not the previously mentioned negatively charged phospholipid membranes) involving the plasma proteins high molecular weight kininogen and plasma kallikrein (contact route). Although FXII is activated by a variety of poly-anions, including constituents of subendothelial matrix (glycosaminoglycans and collagens), sulfatides, nucleosomes and nonphysiological materials (glass, ellagic acid, kaolin, and silica), the *in vivo* contribution of these compounds to coagulation activity remains poorly understood [12, 13].

Ultimately, thrombin converts soluble fibrinogen into fibrin and activates factor XIII (FXIII) which in turn covalently cross-links the soluble fibrin strands into an insoluble fibrin clot. The formation of thrombin is downregulated through three pathways: (i) the tissue factor inhibitor pathway (TFPI) which inhibits the TF: activated FVII (FVIIa): activated FX (FXa) complex, (ii) antithrombin (AT) inhibiting mainly FXa and thrombin, and (iii) the protein C pathway. Protein C is activated by thrombin and utilizes protein S as a cofactor; activated protein C (APC) inhibits the activated forms of cofactors V (Va) and VIIIa through proteolytic inactivation.

Relationship between coagulation and inflammation

Inflammation and coagulation are closely associated and it has long been known that inflammation can lead to activation of the coagulation system. This interaction points to another evolutionary conserved role of blood coagulation, i.e. protection against invading bacteria. The generation of fibrin may serve to wall off bacteria protecting the organism against systemic dissemination of infection. Acute inflammation, as a response to infection or trauma, results in a systemic activation of blood coagulation [14, 15] through TF-mediated thrombin generation, down regulation of physiological anticoagulant mechanisms and

inhibition of fibrinolysis. The resulting hypercoagulability (increased tendency to form a fibrin clot) increases the tendency to thrombosis, but also triggers intracellular signaling processes via activation of protease activated receptors (PARs) by several proteases including thrombin. PAR-mediated cellular effects comprise inflammation and angiogenesis and these effects may have important effects on complex processes like atherosclerosis [16]. PM can provoke an inflammatory response in the lungs, which consequently results in hypercoagulability since prothrombotic and inflammatory cytokines are released into the circulation.

PM10 particles and haemostasis

Air borne PM is currently divided in three types, that are classified according to diameter (PM10, <10 μm; PM2.5, <2.5 μm; ultrafine particles (UFPs), <0.1 μm). These three types are all, although in different ways, involved in the mechanisms that lead towards alterations in haemostasis. The PM10 particles affect the lungs in different ways, depending on their actual size. While PM10 particles cannot reach the most distal lung segments PM2.5, UFPs are able to penetrate the alveoli with far greater efficiency than the PM10 particles [17]. Studying the potential procoagulant effects of PM10, Baccarelli et al. reported that the prothrombin time (PT) shortens at elevated ambient air concentrations of PM10 and gaseous pollutants, mainly carbon monoxide (CO) and nitrogen dioxide (NO_2) , in the 30 days before blood sampling in a cohort study (1218 individuals). In this study, there were neither effects on activated partial thromboplastin time (aPTT), nor on antigen and/or functional levels of fibrinogen, AT, protein C, and protein S (free or total) [18]. Shortening of the PT may be related to increased levels of coagulation proteins, particularly FVIIa, and /or increased activity of the extrinsic coagulation route. In another paper by the same authors, it was reported that exposure to PM10 increases the risk of venous thrombosis [19]. Depending on the ambient concentration of PM10, the relative risk of deep vein thrombosis was increased from 1.7 to more than 10 in patients living in the Lombardy region of Italy. In this study, a shortened PT was associated with increased PM exposure in the subjects suffering from a deep venous thrombosis, whereas the aPTT was not affected. In a recent longitudinal study including 40 healthy volunteers in whom platelet aggregation, thrombin generation and plasma levels of fibrinogen and C reactive protein (CRP) were measured 13 times over a period of 1 year, we observed a positive association between air pollution and platelet aggregation, as well as with the overall coagulation assessed by thrombin generation, without any evidence of systemic inflammation [20]. In this study, we investigated direct (within 24 hours before blood sampling), as well as

indirect effects (within 24-96 hours before blood sampling) of environmental exposure to PM10, CO, nitrogen monoxide (NO), NO_2 and O_3 on blood haemostasis. Fibrinogen and CRP levels did not reveal an inflammatory effect of air pollution. Other studies failed to show clear pro-inflammatory or procoagulant effects of PM10 studying a range of markers including CRP, Il-6, fibrinogen, AT, factors II, VII, VIII and X [21, 22]. However, none of these observational studies explain the mechanisms that may be involved in the hypercoagulable response that occurs upon exposure to PM10.

Experimental studies provide some clues as to the mechanisms involved. Gilmour et al. showed that PM10 affects macrophages, epithelial and endothelial cell function to favor blood coagulation via induction of TF activity and inhibition of fibrinolysis pathways *in vitro* [23]. The animal studies performed with PM10 suggest that these particles cause a pulmonary inflammatory reaction with associated oxidative stress and tissue damage [23, 24]. Animal studies by our group suggest a dose dependent increase in TF and decrease in thrombomodulin (TM) in the lungs of spontaneously hypertensive rats at 4 and 48 hours after PM10 exposure [25]. Mutlu et al. reported a reduced bleeding time, reduced PT and aPTT and increased levels of FVIII, fibrinogen, prothrombin, FX and thrombin-antithrombin (TAT) complexes 24 hours after exposure of C57BL6/J mice to PM10 [26]. Additionally, none of these changes were observed in IL-6 deficient mice, suggesting an inflammation driven disbalance in hemostasis. Depletion of macrophages by liposomal clodranate also resulted in similar null effects. Together, these data suggest that exposure to PM10 results in a dose- and time-dependent inflammation (IL-6) driven activation of coagulation by increasing TF and diminishing TM activities, possibly mediated through macrophages.

PM2.5 and UFPs particles and haemostasis

In general, the studies in which humans and animals were exposed to PM2.5 and UFPs have not explored the same range of coagulation markers discussed for PM10 above. Most investigated parameters include vWF antigen and activity, D-dimer (a fibrin degradation product), protein C, fibrinogen and plasminogen activator inhibitor-1 (PAI-1). Carlsten et al. reported a decrease in vWF antigen with a lag of 7 hours and also a decrease in PAI-1 activity with a lag of 22 hours after exposure to diesel exhaust derived PM2.5 particles in individuals with the metabolic syndrome [27]. Additionally, there was no change in D-dimer. In another study, the same investigators found a decrease in D-dimer with a lag of 6 hours after exposure to filtered air only and a decrease in PAI-1 activity after exposure to filtered air and diesel

exhaust derived PM2.5 particles in healthy human subjects by using the same experimental setup [28]. Both studies from the same group suggest no procoagulant effects, rather a reduced tendency to clot. However, exposure of healthy humans to wood smoke containing high concentrations of PM2.5 particles increases FVIII activity in plasma after 20 hours and FVIII/vWF ratio after 15 minutes, 3 and 20 hours. On the other hand, FVII, vWF activity, D-dimer, and fibrinogen levels were unaffected [29]. In comparison to these studies, Riediker et al. reported that PM2.5 originating from speed-changing traffic was associated with a slight increase in vWF activity and an 11% reduction in protein C concentrations in patrol officers by monitoring the pollutants in their cars during 4 days while working the 3 PM to midnight shift [30]. Taking into account that vWF and FVIII are stored in endothelial cells and released into the circulation upon endothelial cell activation, one must assume that PM2.5 particles may cause endothelial cell activation. Exposure to UFPs also increases vWF activity with a lag of 2, 3 and 4 days after exposure in coronary heart disease patients [31]. On the other hand, another study did not show any procoagulant effects at 6 and 24 hours after exposure of patients with mild chronic obstructive pulmonary disease to diesel UFPs [32]. Finally, prothrombin activation peptide fragment 1 and 2 (F1+2) was increased in association with exposure to PM in a cohort of 57 patients with coronary heart disease. Activation of coagulation was mainly associated with elevated UFP levels, although no association with fibrinogen was found [33]. In all these studies one has to keep in mind that subjects with chronic inflammatory diseases like Chronic obstructive pulmonary disease (COPD) or coronary artery disease may already have upregulated antioxidant enzymes or other protective mechanisms that may in part blunt their inflammatory responses and protect against additional insults of PM exposure.

In general, experimental studies have been more straightforward in determining specific pro- and anticoagulant mechanisms after exposure to PM. In spite of that, there appears to be a number of differences in outcomes, most likely due to differences in PM source and dose used as well as a time dependency of the experimental setup.

Platelets and particles

One of the mechanisms underlying the prothrombotic effects of PM involves increased platelet activation and aggregation [34]. However, it is not yet clear what the exact pathways are that lead from inhalation of PM to platelet activation. Obviously, the PM-mediated effects on platelets are exerted across the lung-blood barrier, either directly by PM after translocation into blood, or indirectly via secondary mediators. It is important to consider the time-frame

within which these effects take place. Previously, it has been shown that exposure to traffic, and concomitantly to combustion-generated nanopartiles, is associated with the onset of myocardial infarction already within 1h [35]. Given the role of platelets in hemostasis to rapidly detect sites of vascular injury, it has been postulated that these acute (short-term) effects of PM might reside in direct triggering of platelet receptors through binding to exogenous surfaces of translocated UFPs. Scanning electron microscopy examinations of human thrombi that were captured by vena cava filters have revealed that foreign nanosized particles are incorporated in these thrombi. This suggests that PM might actively participate in the formation of platelet aggregates serving as core components, a process termed platelet agglutination [36]. Nemmar et al. have demonstrated in a hamster model that already 1h after exposure to diesel exhaust particles (DEP), platelet activation and aggregation is increased. However, these observations were suggested to involve the release of inflammatory intermediates histamine and neutrophil-derived elastase, rather than the direct triggering of platelets [37, 38]. In addition, Khandoga et al. demonstrated in mice that within 2h after intra-arterial infusion of UFPs, platelets accumulate on the endothelium [39].Immuno-histochemical evaluation of the treated vessels revealed fibrinogen and vWF-staining on these endothelial cells, which might explain platelet adhesion to the vessel wall and the local initiation of thrombus formation. Other mechanisms leading to platelet activation involve the effects of PM-induced lung inflammation. This inflammation is characterized by the release of TF-bearing microparticles from activated leucocytes and platelets [40]. In turn, these microparticles mediate the cross talk between platelets and other inflammatory cells and stimulate local coagulation by concentrating TF and other clotting factors on the surfaces of these platelets [41, 42].

Based on the results of these previous studies, we also studied the relationship between air pollution and platelet aggregation in the biological variation study, that we mentioned previously [20]. We observed positive associations between local ambient concentrations of air pollutants PM10, CO, NO, NO_2 and O_3 that were measured within 1 to 4 days before blood sampling, and adenosine diphosphate (ADP)-induced platelet aggregation that was performed in plasma samples collected throughout the one-year study period. Also, indirect effects on thrombin generation were observed leading to increased endogenous thrombin potential and peak thrombin generation, which suggests that increased platelet aggregation is accompanied by hypercoagulability. No direct effects of air pollutants on platelet aggregation (association with air pollution levels in the 24 hr preceding the blood sampling) were observed, which we have confirmed *in vitro* by the absence of alterations in ADP-induced light-transmittance and

whole-blood aggregometry after incubation (0-2h) of platelet-rich plasma or whole blood, respectively, with different doses and types of PM. As mentioned before, we did not find any effects of the studied air pollutants on the plasma levels of inflammatory markers fibrinogen and CRP, which suggests that the observed effects on platelet aggregation do not rely on changes in these inflammatory mediators. Conversely, we instilled either UFPs collected in Mexico City or DEP in mice, and afterward, cells in broncoalveolar lavage (BAL) of lungs were stained with Diff-Quick staining 24 h after instillation. Uptake of UFPs or DEP by macrophages in mice compared to saline instilled or sham mice is obviously seen in histological picture (Fig. 1.1). This however suggests the possibility that other inflammatory markers released from macrophages, such as IL-6 or tumor necrosis factor alpha (TNF-α), might be involved [26,43].

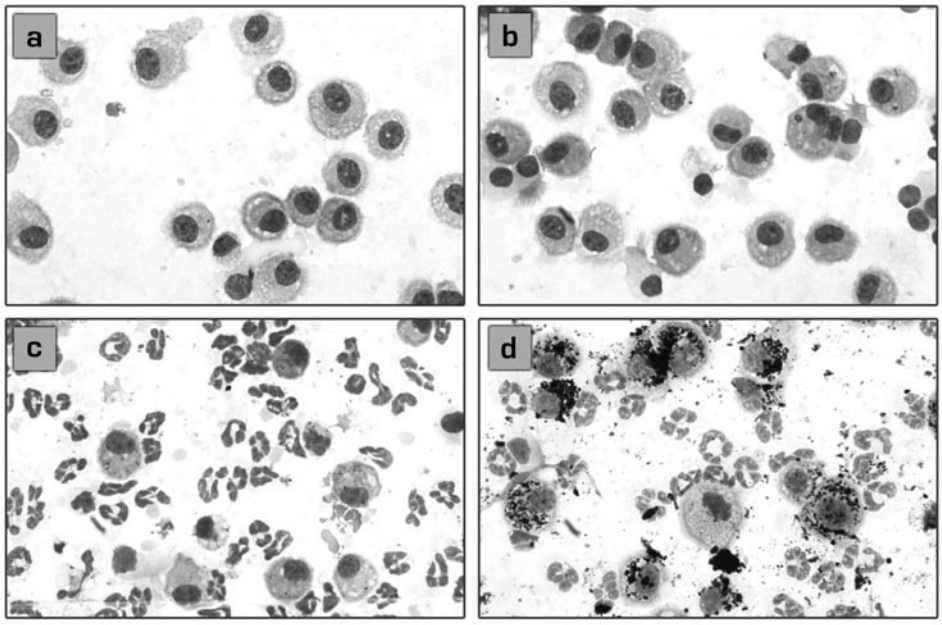


Figure 1.1. Diff-Quick staining of cells in brocho-alveolar lavage of lungs of Sham mice (a) and of mice 24 h after the intratracheal instillation with vehicle (saline, containing 0.1%Tween) (b), with 100 μg utrafine particulate matter (UFPs), collected in Mexico City (c) or with 100 μg diesel exhaust particles (DEP) (d), suspended in vehicle. Note the increased inflammation for the particulate matter and the ingestion by lung macrophages of the black particles present in diesel exhaust.

Extrapulmonary translocation of UFPs

Intratracheally (i.t.) instilled UFPs can pass from the lungs into the blood circulation in hamsters [44]. This was demonstrated by using 80 nm albumin-nanocolloid particles labeled

radioactively with technetium-99, as a model of UFPs, and via the study of their distribution in the blood and other organs after their i.t. administration in the hamster. A substantial fraction of ^{99m}Tc-albumin was found to diffuse rapidly (within minutes and up to one hour) from the lungs into the systemic circulation [44]. In addition to our findings, others have also reported extrapulmonary translocation of UFPs after i.t. instillation or inhalation in other animal species [45-47]. The reported amount of UFPs translocating into blood and extrapulmonary organs differed, however, amongst these studies. Nevertheless, translocation was also found to occur to tissues in the systemic compartment, following intranasal delivery of polystyrene microparticles of 1.1 μm [48].

Recent morphological findings have illustrated that inhaled particles are transported into the pulmonary capillary space, presumably by transcytosis [49-51]. In *ex vivo* models of isolated perfused lungs of rat [52] and rabbit [37], in the absence of lymph flow and inflammatory cell recruitment, UFPs can translocate from the lung into the circulation and *visa versa*, upon pharmacological modulation (H_2O_2 or vascularly administered histamine), increasing the pulmonary microvascular permeability.

Even though several recent animal exposure studies report translocation of UFPs in humans, evidence for the existence of translocation of UFPs from the lungs into the blood circulation is limited and the data are conflicting. We studied the distribution of radiolabelled ultrafine carbon particles, commonly known as "Technegas", after their inhalation by non-smoking healthy human volunteers [53]. Individualized particles had a diameter in the order of 5 to 10 nm, as confirmed by electron microscopy. Radioactivity was detected in blood already after 1 minute, reaching a maximum between 10 and 20 minutes, remaining at this level up to 60 minutes. This radioactivity appeared to be largely particle-bound, as assessed by thin layer chromatography. Gamma camera images showed substantial radioactivity over the liver and other areas of the body. The presence of radioactivity in the liver is compatible with an accumulation of particles in Kupffer cells, known to occur with colloidal particles [54]. In agreement with these findings, Kawakami *et al*. reported the presence of radioactivity in blood immediately after inhalation of ^{99m}Tc-technegas in human volunteers [55]. In contrast, recent studies investigating the translocation of UFPs (10 nm in diameter, yet rapidly forming aggregates of 100 nm in diameter in the inhaled aerosol) administered as a technetium-99m–labelled aerosol in human volunteers reported radioactivity mainly localized in the lung (95.6±1.7%), with no radioactivity found over the liver [56, 57]. Moreover, the nature of the radioactivity found in blood (4.4 %) consisted mainly of pertechnetate, as analyzed by thin layer chromatography. Further studies are required to resolve these discrepancies in man.

Effects of particles on thrombogenesis and platelet activation in experimental animal models

As outlined above, exposure to UFPs is associated with the rapid occurrence of myocardial infarction [35, 58]. In view of the important role of thrombosis in myocardial infarction, several animal studies were performed in hamsters and mice to assess the effects of particles on thrombosis, as a relevant cardiovascular endpoint. To mimic the existence of minimal vascular disease in man, in these models a mild local endothelial lesion was produced in blood vessels, by radicals formed upon decomposition of Rose Bengal, upon its irradiation with green light in a peripheral vessel. Hence, flow-controlled venous or arterial thrombi form, which can be followed online via transillumination and quantified by image analysis [59]. The intensity of thrombosis can be linked to the degree of vessel wall injury and to activation of hemostasis pathways.

Initially, well-characterized and chemically relatively inert polystyrene particles of 60 nm diameter were tested as a model of UFPs [60]. Moreover, polystyrene UFPs enabled easy surface modification, to allow for comparison between particles with neutral, negative or positive surface charges, mimicking the fact that most ambient particles are charged. Haemostasis was affected by the intravenous injection in hamsters of such UFPs, depending on their surface properties. Positively charged amine-modified particles led to a marked increase in prothrombotic tendency resulting, at least in part, from platelet activation [60]. Correspondingly, positively charged amine-modified polystyrene particles induced platelet aggregation *in vitro* and strongly increased the ADP-triggered aggregation in a dose-dependent manner, thus providing a mechanistic basis for the enhanced thrombogenicity observed *in vivo*. Confocal fluorescent microscopy analysis demonstrated that individual platelets were strongly stained by amine-polystyrene particles but not by the carboxylate-polystyrene particles. In subsequent experiments, using i.t. administered ultrafine polystyrene particles, in addition to causing neutrophil influx in the lung, the positively charged particles (60 nm) were also capable of enhancing the peripheral thrombogenicity, when analysed one hour after instillation [61]. In parallel, we found that 400-nm positive particles also caused pulmonary inflammation at one hour, but they did not enhance *in vivo* thrombosis at all, one hour after i.t. instillation. These findings clarified that the enhanced peripheral thrombogenicity at 1h could not be explained solely by pulmonary inflammation, implying that direct passage of UFPs (but not the larger 400 nm particles) and activation of circulating

platelets contributed to the development of an extrapulmonary and peripheral prothrombotic tendency.

Subsequently, also Silva et al. assessed in a rat model of ear vein thrombosis the effects of i.v. and i.t. administration of amine- and carboxylate-modified polystyrene particles of similar diameter [62]. These authors also found a dose-dependent enhanced thrombogenicity for positively charged UFPs but not for negatively charged UFPs of the same size. Likewise, Khandoga et al. observed that intra-arterial injection of ultrafine carbon particles led to prothrombotic changes in mice [39].

The same hamster model of photochemically induced vascular thrombosis was also applied to study the effects of real pollutant particles, i.e. DEP on thrombosis, when given i.t.. In this model, DEP caused a dose-dependent enhanced peripheral venous and arterial thrombogenicity, one hour after their deposition in the lungs [38]. As flow-dependent thrombosis in the model depends on primary hemostatic activation (platelet adhesion and aggregation), the role of platelets during thrombus formation was assessed ex vivo, in time-wise blood samples from hamsters exposed to DEP. Platelet function was tested in the platelet function analyzer (PFA-100), which device measures platelet activation and aggregation in a shear stress–dependent way [63], by recording a flow pattern and a closure time. In blood samples from hamsters i.t. instilled with DEP, platelet activation was apparent already 30 minutes after instillation. Additional controls showed that the direct addition of DEP to hamster blood *in vitro* already caused platelet activation within 5 min, with as little as 0.5 μg/ml of DEP [38].

Elevation of histamine levels has been correlated with the onset of myocardial infarction [64-66], but the evidence linking exposure to DEP and release of histamine is more abundant in the context of airway allergic and inflammatory processes. Thus, an increase in mast cell numbers in the submucosa and elevated bronchoalveolar lavage (BAL) histamine levels were observed, in humans, 6 hours after exposure to diesel exhaust particles (DEP) [67]. DEP have also been demonstrated to directly degranulate mast cells and to increase histamine levels and symptom severity in humans [68, 69]. Therefore histamine was measured at different time points (1, 6 and 24 h) after i.t. instillation of DEP, both in the respiratory tract and in the peripheral circulation and the effects of pre-treatment with a histamine receptor-1 blocking agent (diphenhydramine) were investigated, on pulmonary inflammation and vascular thrombogenicity. Pre-treatment with diphenhydramine attenuated both pulmonary inflammation and the prothrombotic effect at 6 h and 24 h. At one hour, only the pulmonary inflammation was diminished, whereas platelet activation and the enhanced thrombogenicity

were not affected. In agreement with the statements made above, these early effects are compatible with direct platelet activation by DEP (or their constituents) having penetrated into the circulation [70]. In subsequent experiments, we found, 24 h after DEP exposure, that pre-treatment with dexamethasone or with cromoglycate blocked the DEP-induced pulmonary inflammation, prothrombotic events and histamine release in BAL and plasma. Therefore, the systemic inflammatory and prothrombotic effects observed 24 h after DEP administration seem secondary to lung inflammation; they can be prevented by mast cell stabilization [71]. Of note, we did not find increased levels of plasma vWF, which is a marker of endothelial activation, after DEP administration [71].

The relationship between pulmonary inflammation and thrombotic complications has been studied further, using the established model of sustained pulmonary inflammation induced by silica particles. In the hamster, we demonstrated that the i.t. instillation of silica particles leads to significant dose-dependent increases of macrophage and neutrophil numbers in BAL and the development of a prothrombotic tendency in circulating blood [72]. By specifically depleting lung macrophages with clodronate-liposomes, both the influx of PMN in BAL and the peripheral thrombotic tendency were abrogated. The depletion of circulating neutrophils and monocytes by cyclophosphamide also abolished both the cellular influx in BAL and the peripheral thrombotic tendency, despite normal numbers of lung macrophages. Moreover, silica caused an increase in neutrophil elastase activity in plasma. These findings uncover pulmonary macrophage–neutrophil cross-talk releasing neutrophil elastase into the blood circulation. Elastase, by participating in the activation of circulating platelets, may then predispose platelets to initiate thrombotic events on mildly damaged vasculature.

The more recent application of photochemically induced thrombosis in the carotid artery in the mouse opened the possibility of studying biological interactions more precisely, owing to the larger number of reagents available for the mouse and the availability of genetically modified mice. During i.t. instillation of carbon nanotubes in the mouse, we established a critical role for platelet P-selectin in mediating systemic inflammatory reactions, accelerating thrombus formation [73]. Lung inflammation was found to lead to enhanced heteroconjugate formation between circulating platelets and monocytes, respectively granulocytes, in turn resulting in leukocyte activation, and production of TF positive microvescicles in the circulation. Neutralizing P-selectin with a monoclonal antibody abrogated heteroconjugate formation and the production of microvesicles. At the same time the peripheral thrombogenicity was eliminated. These findings illustrated the critical role of P-selectin in transmission of pulmonary inflammation to the systemic circulation.

Conclusions

The majority of experimental and clinical (epidemiological) studies point to an effect of different sources of PM on the blood coagulation system. These effects include alterations in platelet function, coagulation activity and to a lesser effect on fibrinolysis. Two mechanistic scenarios may be operational, and these may not be mutually exclusive:

a. ***Inflammation driven scenario***;

   In this scenario, exposure to PM provokes an inflammatory response in the lung with consequent release of pro inflammatory cytokines into the circulation. PM predominantly triggers IL-6 production by alveolar macrophages. The procoagulant response to PM is characterized by increased cellular expression of TF and probably loss of vascular endothelial TM activity. The perturbation of the balance between pro- and anticoagulant activity in organs exposed to PM may thus be a contributing factor to development of cardiovascular disease (Fig. 1.2).

b. ***Direct effect scenario***;

   This hypothesis proposes that inhaled, insoluble, PM2.5 or nanoparticles could rapidly translocate into the circulation, with the potential for direct effects on hemostasis and adverse cardiovascular endpoints. Translocation of inhaled nanoparticles across the alveolar–blood barrier has been demonstrated in animal studies for a range of nanoparticles delivered by inhalation or instillation. Translocation of these particles may also directly activate the contact system of blood coagulation via factor XII activation [74] (Fig. 1.2).

In conclusion, a large variety of both *in vivo* and *in vitro* studies have demonstrated the association between exposure to PM in air pollution and activation or alterations of the haemostatic system. Epidemiologic studies have clearly shown the association between PM exposure and the risk for both venous and arterial thrombosis. Platelet activation and hypercoagulability have been shown in human volunteer and animal studies and both effects appear to be inflammation driven, although the study data are not consistent.

In general however, it seems prudent to conclude that PM may through different mechanisms enhance blood coagulation activity which may contribute to the risk of developing thrombosis. In addition to an effect on venous thrombosis, PM may either by

accelerating atherosclerosis or by stimulating a platelet dependent clotting response, also induces the risk of arterial thrombosis. These prothrombotic effects of PM are likely to contribute to the health hazards involved in environmental exposure to PM.

Acknowledgements

These studies were financially supported by the ECAT foundation (Leiden, the Netherlands). Leuven nl OT/07/066 and the CMVB is supported by the 'Excellentie financiering KULeuven' (EF Ž 05 Ž 013) including the Netherlands Heart Foundation with a grant number of 2006B064.

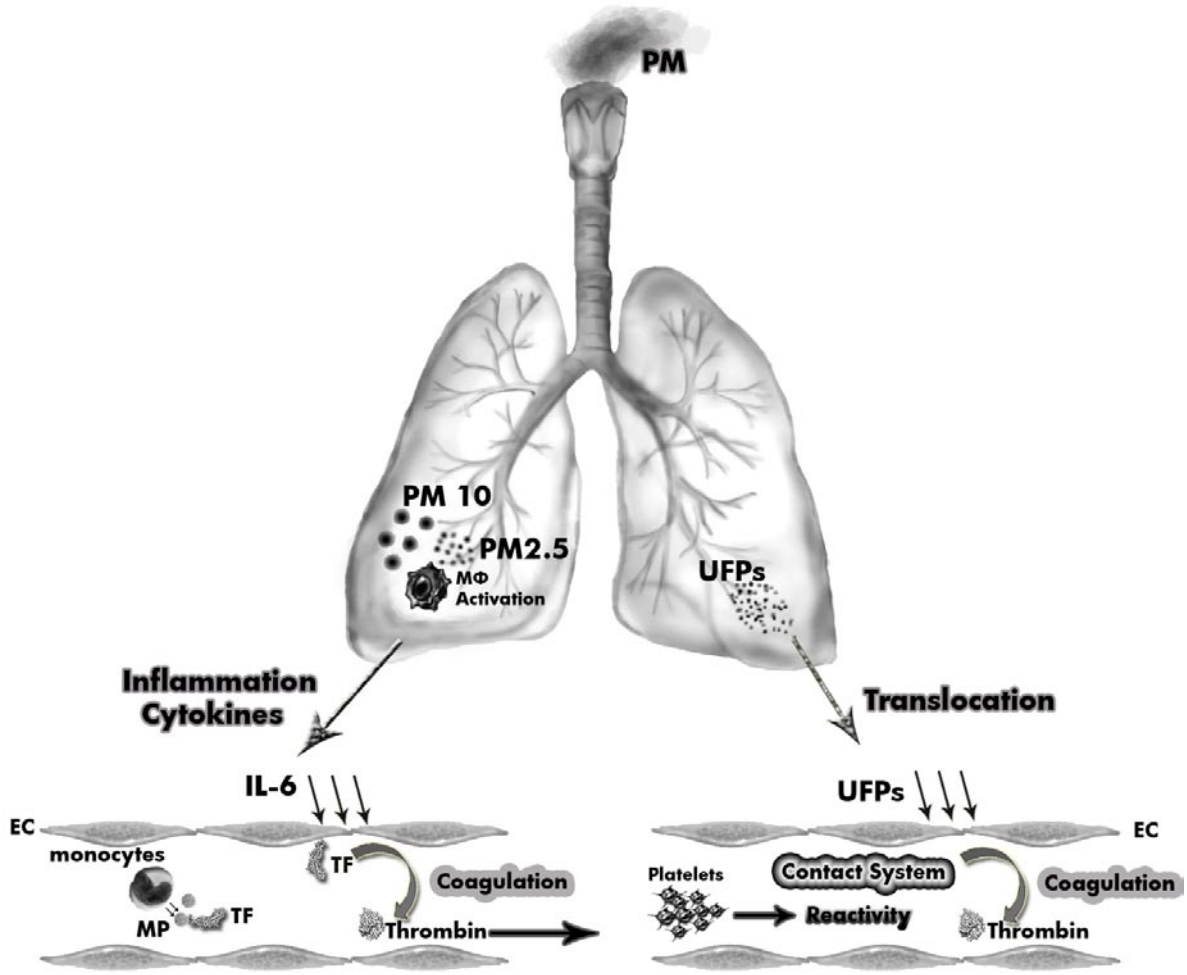


Figure 1.2. ***A schematic illustration of proinflammatory and procoagulant effects of particulate matter (PM)***

Once inhaled, PM may cause systemic harmful effects either mediated through pulmonary inflammatory effects, or through direct translocation of PM into the circulation. PM10 (less than 10μm particles) and PM2.5 (less than 2.5μm particles) induce macrophage (MΦ) activation in the lung and MΦs secrete proinflammatory cytokines such as interleukin – 6 (IL-6). The production of IL-6 induces tissue factor (TF) expression on endothelial cells (EC). However, monocytes represent a large pool of circulating precursors that can differentiate into MΦs which are also triggered by IL-6. Furthermore, the differentiation process of monocytes to MΦs releases TF rich microparticles (MP) into the blood. Expression of TF on endothelial cells or on MP triggers blood coagulation via activation of the extrinsic pathway which results in thrombin generation. Additionally, ultrafine particles (UFPS; <0.15μm) may directly translocate into circulation and alternatively, thrombin may be generated through PM mediated contact activation. Thrombin is also one of the agonists of platelet reactivity.

References

1 Delfino RJ, Sioutas C, Malik S. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. *Environ Health Perspect*. 2005; **113**: 934-46.

2 Kunzli N, Tager IB. Air pollution: from lung to heart. *Swiss Med Wkly*. 2005; **135**: 697-702.

3 Mills NL, Tornqvist H, Robinson SD, Gonzalez MC, Soderberg S, Sandstrom T, Blomberg A, Newby DE, Donaldson K. Air pollution and atherothrombosis. *Inhal Toxicol*. 2007; **19 Suppl 1**: 81-9.

4 Gold DR, Litonjua A, Schwartz J, Lovett E, Larson A, Nearing B, Allen G, Verrier M, Cherry R, Verrier R. Ambient pollution and heart rate variability. *Circulation*. 2000; **101**: 1267-73.

5 Riediker M, Cascio WE, Griggs TR, Herbst MC, Bromberg PA, Neas L, Williams RW, Devlin RB. Particulate matter exposure in cars is associated with cardiovascular effects in healthy young men. *Am J Respir Crit Care Med*. 2004; **169**: 934-40.

6 Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet*. 1995; **345**: 176-8.

7 Seaton A, Soutar A, Crawford V, Elton R, McNerlan S, Cherrie J, Watt M, Agius R, Stout R. Particulate air pollution and the blood. *Thorax*. 1999; **54**: 1027-32.

8 O'Neill MS, Veves A, Sarnat JA, Zanobetti A, Gold DR, Economides PA, Horton ES, Schwartz J. Air pollution and inflammation in type 2 diabetes: a mechanism for susceptibility. *Occup Environ Med*. 2007; **64**: 373-9.

9 O'Neill MS, Veves A, Zanobetti A, Sarnat JA, Gold DR, Economides PA, Horton ES, Schwartz J. Diabetes enhances vulnerability to particulate air pollution-associated impairment in vascular reactivity and endothelial function. *Circulation*. 2005; **111**: 2913-20.

10 Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol*. 2008; **8**: 776-87.

11 Spronk HM, Govers-Riemslag JW, ten Cate H. The blood coagulation system as a molecular machine. *Bioessays*. 2003; **25**: 1220-8.

12 Muller F, Renne T. Novel roles for factor XII-driven plasma contact activation system. *Curr Opin Hematol*. 2008; **15**: 516-21.

13 Schousboe I. Pharmacological regulation of factor XII activation may be a new target to control pathological coagulation. *Biochem Pharmacol*. 2008; **75**: 1007-13.

14 Levi M, Keller TT, van Gorp E, ten Cate H. Infection and inflammation and the coagulation system. *Cardiovasc Res*. 2003; **60**: 26-39.

15 ten Cate H, Falanga A. Overview of the postulated mechanisms linking cancer and thrombosis. *Pathophysiol Haemost Thromb*. 2008; **36**: 122-30.

16 Borissoff JI, Spronk HM, Heeneman S, ten Cate H. Is thrombin a key player in the 'coagulation-atherogenesis' maze? *Cardiovasc Res*. 2009; **82**: 392-403.

17 Harrison RM, Yin J. Particulate matter in the atmosphere: which particle properties are important for its effects on health? *Sci Total Environ*. 2000; **249**: 85-101.

18 Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost*. 2007; **5**: 252-60.

19 Baccarelli A, Martinelli I, Zanobetti A, Grillo P, Hou LF, Bertazzi PA, Mannucci PM, Schwartz J. Exposure to particulate air pollution and risk of deep vein thrombosis. *Arch Intern Med*. 2008; **168**: 920-7.

20 Rudez G, Janssen NA, Kilinc E, Leebeek FW, Gerlofs-Nijland ME, Spronk HM, ten Cate H, Cassee FR, de Maat MP. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect*. 2009; **117**: 995-1001.

21 Brauner EV, Forchhammer L, Moller P, Barregard L, Gunnarsen L, Afshari A, Wahlin P, Glasius M, Dragsted LO, Basu S, Raaschou-Nielsen O, Loft S. Indoor particles affect vascular function in the aged: an air filtration-based intervention study. *Am J Respir Crit Care Med*. 2008; **177**: 419-25.

22 Scharrer E, Hessel H, Kronseder A, Guth W, Rolinski B, Jorres RA, Radon K, Schierl R, Angerer P, Nowak D. Heart rate variability, hemostatic and acute inflammatory blood parameters in healthy adults after short-term exposure to welding fume. *Int Arch Occup Environ Health*. 2007; **80**: 265-72.

23 Gilmour PS, Morrison ER, Vickers MA, Ford I, Ludlam CA, Greaves M, Donaldson K, MacNee W. The procoagulant potential of environmental particles (PM10). *Occup Environ Med*. 2005; **62**: 164-71.

24 Brown DM, Hutchison L, Donaldson K, Stone V. The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *Am J Physiol Lung Cell Mol Physiol*. 2007; **292**: L1444-51.

25 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.

26 Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest*. 2007; **117**: 2952-61.

27 Carlsten C, Kaufman JD, Trenga CA, Allen J, Peretz A, Sullivan JH. Thrombotic markers in metabolic syndrome subjects exposed to diesel exhaust. *Inhal Toxicol*. 2008; **20**: 917-21.

28 Carlsten C, Kaufman JD, Peretz A, Trenga CA, Sheppard L, Sullivan JH. Coagulation markers in healthy human subjects exposed to diesel exhaust. *Thromb Res*. 2007; **120**: 849-55.

29 Barregard L, Sallsten G, Gustafson P, Andersson L, Johansson L, Basu S, Stigendal L. Experimental exposure to wood-smoke particles in healthy humans: effects on markers of inflammation, coagulation, and lipid peroxidation. *Inhal Toxicol*. 2006; **18**: 845-53.

30 Riediker M, Devlin RB, Griggs TR, Herbst MC, Bromberg PA, Williams RW, Cascio WE. Cardiovascular effects in patrol officers are associated with fine particulate matter from brake wear and engine emissions. *Part Fibre Toxicol*. 2004; **1**: 2.

31 Yue W, Schneider A, Stolzel M, Ruckerl R, Cyrys J, Pan X, Zareba W, Koenig W, Wichmann HE, Peters A. Ambient source-specific particles are associated with prolonged repolarization and increased levels of inflammation in male coronary artery disease patients. *Mutat Res*. 2007; **621**: 50-60.

32 Blomberg A, Tornqvist H, Desmyter L, Deneys V, Hermans C. Exposure to diesel exhaust nanoparticles does not induce blood hypercoagulability in an at-risk population. *J Thromb Haemost*. 2005; **3**: 2103-5.

33 Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med*. 2006; **173**: 432-41.

34 Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, Sandstrom T, Blomberg A, Newby DE. Adverse cardiovascular effects of air pollution. *Nat Clin Pract Cardiovasc Med*. 2009; **6**: 36-44.

35 Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, Lowel H. Exposure to traffic and the onset of myocardial infarction. *N Engl J Med*. 2004; **351**: 1721-30.

36 Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol*. 2005; **146**: 882-93.

37 Nemmar A, Hamoir J, Nemery B, Gustin P. Evaluation of particle translocation across the alveolo-capillary barrier in isolated perfused rabbit lung model. *Toxicology*. 2005; **208**: 105-13.

38 Nemmar A, Hoet PH, Dinsdale D, Vermylen J, Hoylaerts MF, Nemery B. Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. *Circulation*. 2003; **107**: 1202-8.

39 Khandoga A, Stampfl A, Takenaka S, Schulz H, Radykewicz R, Kreyling W, Krombach F. Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice in vivo. *Circulation*. 2004; **109**: 1320-5.

40 Hrachovinova I, Cambien B, Hafezi-Moghadam A, Kappelmayer J, Camphausen RT, Widom A, Xia L, Kazazian HH, Jr., Schaub RG, McEver RP, Wagner DD. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat Med*. 2003; **9**: 1020-5.

41 Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. *Blood*. 2000; **95**: 1317-23.

42 Namba M, Tanaka A, Shimada K, Ozeki Y, Uehata S, Sakamoto T, Nishida Y, Nomura S, Yoshikawa J. Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood. *Arterioscler Thromb Vasc Biol*. 2007; **27**: 255-6.

43 Ruckerl R, Greven S, Ljungman P, Aalto P, Antoniades C, Bellander T, Berglind N, Chrysohoou C, Forastiere F, Jacquemin B, von Klot S, Koenig W, Kuchenhoff H, Lanki T, Pekkanen J, Perucci CA, Schneider A, Sunyer J, Peters A, Group AS. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environ Health Perspect*. 2007; **115**: 1072-80.

44 Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med*. 2001; **164**: 1665-8.

45 Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A*. 2002; **65**: 1513-30.

46 Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A*. 2002; **65**: 1531-43.

47 Takenaka S, Karg E, Roth C, Schulz H, Ziesenis A, Heinzmann U, Schramel P, Heyder J. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environ Health Perspect*. 2001; **109 Suppl 4**: 547-51.

48 Eyles JE, Bramwell VW, Williamson ED, Alpar HO. Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration. *Vaccine*. 2001; **19**: 4732-42.

49 Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect*. 2005; **113**: 1555-60. 50 Kapp N, Kreyling W, Schulz H, Im Hof V, Gehr P, Semmler M, Geiser M. Electron energy loss spectroscopy for analysis of inhaled ultrafine particles in rat lungs. *Microsc Res Tech*. 2004; **63**: 298-305.

51 Kato T, Yashiro T, Murata Y, Herbert DC, Oshikawa K, Bando M, Ohno S, Sugiyama Y. Evidence that exogenous substances can be phagocytized by alveolar epithelial cells and transported into blood capillaries. *Cell Tissue Res*. 2003; **311**: 47-51.

52 Meiring JJ, Borm PJ, Bagate K, Semmler M, Seitz J, Takenaka S, Kreyling WG. The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung. *Part Fibre Toxicol*. 2005; **2**: 3.

53 Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation*. 2002; **105**: 411-4.

54 Simon BH, Ando HY, Gupta PK. Circulation time and body distribution of 14C-labeled amino-modified polystyrene nanoparticles in mice. *J Pharm Sci*. 1995; **84**: 1249-53.

55 Kawakami K, Iwamura A, Goto E, Mori Y, Abe T, Hirasawa Y, Ishida H, Shimada T, Tominaga S. [Kinetics and clinical application of 99mTc-technegas]. *Kaku Igaku*. 1990; **27**: 725-33.

56 Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, Macnee W, Millar AM, Donaldson K, Newby DE. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *Am J Respir Crit Care Med*. 2006; **173**: 426-31.

57 Moller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, Haussinger K, Kreyling WG. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. *Am J Respir Crit Care Med*. 2008; **177**: 426-32.

58 Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. *Circulation*. 2001; **103**: 2810-5.

59 Kawasaki T, Kaida T, Arnout J, Vermylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. *Thromb Haemost*. 1999; **81**: 306-11.

60 Nemmar A, Hoylaerts MF, Hoet PH, Dinsdale D, Smith T, Xu H, Vermylen J, Nemery B. Ultrafine particles affect experimental thrombosis in an in vivo hamster model. *Am J Respir Crit Care Med*. 2002; **166**: 998-1004.

61 Nemmar A, Hoylaerts MF, Hoet PH, Vermylen J, Nemery B. Size effect of intratracheally instilled particles on pulmonary inflammation and vascular thrombosis. *Toxicol Appl Pharmacol*. 2003; **186**: 38-45.

62 Silva VM, Corson N, Elder A, Oberdorster G. The rat ear vein model for investigating in vivo thrombogenicity of ultrafine particles (UFP). *Toxicol Sci*. 2005; **85**: 983-9.

63 Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer--PFA-100. *Semin Thromb Hemost*. 1995; **21 Suppl 2**: 106-12.

64 Clejan S, Japa S, Clemetson C, Hasabnis SS, David O, Talano JV. Blood histamine is associated with coronary artery disease, cardiac events and severity of inflammation and atherosclerosis. *J Cell Mol Med*. 2002; **6**: 583-92.

65 Laine P, Kaartinen M, Penttila A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation*. 1999; **99**: 361-9.
66 Zaca F, Benassi MS, Ghinelli M, Trianni M, Vaccarino R, Malavolta E, Muratori M, Lenzi S. Myocardial infarction and histamine release. *Agents Actions*. 1986; **18**: 258-61.
67 Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med*. 1999; **159**: 702-9.
68 Devouassoux G, Saxon A, Metcalfe DD, Prussin C, Colomb MG, Brambilla C, Diaz-Sanchez D. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol*. 2002; **109**: 847-53.
69 Diaz-Sanchez D, Penichet-Garcia M, Saxon A. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol*. 2000; **106**: 1140-6.
70 Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. *Am J Respir Crit Care Med*. 2003; **168**: 1366-72.
71 Nemmar A, Hoet PH, Vermylen J, Nemery B, Hoylaerts MF. Pharmacological stabilization of mast cells abrogates late thrombotic events induced by diesel exhaust particles in hamsters. *Circulation*. 2004; **110**: 1670-7.
72 Nemmar A, Nemery B, Hoet PH, Van Rooijen N, Hoylaerts MF. Silica particles enhance peripheral thrombosis: key role of lung macrophage-neutrophil cross-talk. *Am J Respir Crit Care Med*. 2005; **171**: 872-9.
73 Nemmar A, Hoet PH, Vandervoort P, Dinsdale D, Nemery B, Hoylaerts MF. Enhanced peripheral thrombogenicity after lung inflammation is mediated by platelet-leukocyte activation: role of P-selectin. *J Thromb Haemost*. 2007; **5**: 1217-26.
74 Kilinc E, Van Oerle R, Borissoff JI, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HM. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *J Thromb Haemost*. 2011; **9**: 1359-67.

CHAPTER 1

GENERAL INTRODUCTION

PART II:
PARTICULATE MATTER INDUCED ATHEROSCLEROSIS:
A MECHANISTIC OUTLOOK

Kilinç, E., Borissoff, J.I., Spronk, H.M.H., ten Cate, H.

Submitted

Pioneering studies have documented inhalation of PM as a risk factor for cardiovascular disease including myocardial infarction, arrhythmia, heart failure and stroke [1-4]. In addition to inflammatory, procoagulant and thrombotic effects of PM discussed in part 1, several human and animal studies have shown that exposure to PM enhances atherosclerosis [5-12], as a main cause of cardiovascular disease.

There is abundant evidence documenting a fundamental role for inflammation in atherosclerotic plaque formation and progression [13, 14]. Macrophages, neutrophils, T cells, mast cells and other cells infiltrate the subendotelium at the site of fatty streaks during development of atherosclerotic plaques [13, 15, 16]. Although the mechanisms of air pollution induced atherosclerosis are not yet fully understood, two main hypotheses are proposed with regard to this pro-atherogenic effect of PM. The first concept is that inhaled PM in the lungs causes pulmonary and subsequent systemic inflammation, associated with oxidative stress during phagocytosis of PM by pulmonary macrophages [17, 18]. Oxidative stress ultimately mediates endothelial dysfunction [19]. Endothelial dysfunction, followed by low density lipoprotein (LDL) accumulation, are the first events in the initiation phase of atherosclerosis [20]. However, these mechanisms have not been precisely examined. The second hypothesis considers translocation of ultrafine particles (UFPs; <0.1 μm) into the circulation as a critical pathway to activation of cells through direct interaction of cells, proteins and tissues [21, 22]. In this part, the effects of PM on several aspects related to atherosclerosis will be discussed.

PM induces Oxidative Stress related DNA damage

It is generally accepted that the effect of PM on cellular damage is mainly the result of generation of reactive oxygen species (ROS), especially hydroxyl radicals (HO·), which have a pivotal role in PM–induced adverse health effects [23]. These pathophysiological effects are dependent on the size of particles, and their chemical composition [24]. Beyond the direct pro-oxidative potential of PM, the lung also releases locally and systemically ROS following inhalation of PM [25]. As an outcome of several studies, 8-hydroxy-2′-deoxyguanosine (8-OHdG) has been established as an important specific biomarker of oxidative stress [26, 27]. The interaction of HO• with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-OHdG) [28]. In a cohort study, exposure to fine PM (PM2.5; PM<2.5μm) was found to increase oxidative DNA damage measured by level of the urinary 8-OHdG after their shift ends [29]. Additionally, exposure to metal components such as manganese, nickel, and lead were found positively associated with 8-OHdG in this study. In a supportive *in vitro* study, the different

PMs (PM10; PM<10μm, PM2.5, diesel exhaust particles (DEP), gasoline exhaust particles and wood smoke soot under the same conditions) contributed to the formation of HO· but DEP and PM2.5 caused the most dramatic increase in 8OHdG level with the highest metal content contribution from Fe (II) and V (IV)[27]. Prior to these studies, an experimental mice study suggested an increased formation of 8-OHdG in lung DNA from mice treated with DEP in a dose dependent manner [30]. In a similar experimental setup, carbonaceous particles derived from DEP are suggested to promote the formation of 8-OHdG in mice lungs [31]. One may speculate that these phenomena are the result of the oxygen radicals release during phagocytosis of particles by macrophages, generated by enzymatic or non-enzymatic reactions in the lungs. This could be related to the formation of 8-OHdG and DNA damage.

PM triggered Apoptosis

Apoptosis is an important mechanism by which cells with DNA damage are eliminated without inciting an inflammatory response [32]. The responses to DNA damage will ultimately lead to DNA repair and sustained survival or to controlled cell death [32]. Apoptosis is regulated by either the intrinsic (mitochondrial) or extrinsic (death receptor) pathways. The mitochondrial death pathway mediates apoptosis caused by DNA damage [32, 33]. Experimental evidence suggests PM mediated apoptosis [34-37]. The effect of DEPs on apoptosis was investigated in pulmonary alveolar macrophages or murine and human macrophage cell lines [34]. The key finding was that organic extracts of DEPs induce apoptosis or generate ROS on macrophages in a cell specific manner (only phagocytic cells). PM induced apoptosis is also shown to be dependent on mitochondrial ROS production in alveolar epithelial cells [36] and this initiates the intrinsic apoptotic pathway through the oxidant-sensitive kinase ASK1 and its downstream kinases JNK and p53 [35]. Although p53 modulates apoptosis by complex and incompletely understood mechanisms, it has been shown to be regulated by the BH3 domain-only proteins NOXA and PUMA [38]. However, a mechanism to PM regulated cell apoptosis and lung inflammation by activation of only NOXA protein but not PUMA protein has been recently reported [37]. With respect to PM mediated apoptosis, apoptosis of in particularly macrophages and vascular smooth muscle cells (VSMCs), occurs in different stages of atherosclerotic plaque formation [39].Apoptosis contributes to atherosclerotic plaque growth, lipid core development, plaque rupture and thrombosis but the extent to which apoptosis regulates these processes is unknown [40]. Atherosclerotic plaques tend to rupture at sites of increased macrophage and reduced VSMCs content [41]. There is also recent evidence of mast cell involvement in VSMC death within the fibrous cap [42].

PM and increased Oxidized LDL

At the early stage of atherosclerosis, activation of vascular endothelial cells, adhesion of monocytes and migration of T lymphocytes into the subendothelial space are known events [13, 15, 16]. Oxidized LDL (ox-LDL) leads to the formation of foam cells, a characteristic cell constituent of atherosclerotic lesions [43]. Foam cells secrete proinflammatory cytokines, produce growth factors and express tissue factor [44, 45]. Additionally, ROS production by foam cells enhances leukocyte chemotaxis through vascular endothelium and also regulates VSMC migration and proliferation into the intima. Ox-LDL itself may induce the expression of adhesion molecules such as vascular cell adhesion protein-1 (VCAM-1), inter-cellular adhesion molecule-1 (ICAM-1) and P-selectin, scavenger receptors and matrix metalloproteinases (MMPs). In addition, ox-LDL triggers migration and apoptosis of vascular smooth muscle cells [44, 46].

It has been shown that inhalation of PM in patients with diabetes was associated with increased plasma ox-LDL [47]. Recent animal studies further corroborate this finding. In a hyperlipidemic mouse ($LDLR^{-/-}$ mice) model, exposure to PM2.5 increased the formation of plasma ox-LDL regardless of the dietary regimen (normal or high-fat chow) [48]. Nevertheless, the lipid content of atherosclerotic plaques remained unaffected by PM2.5 exposure. The increased thickness in arterial wall in mice treated with PM2.5 under high fat diet conditions may be attributed to other components such as extracellular matrix production and cellular proliferation. Recently published data consolidate a possible mechanism for PM induced atherosclerosis. Using an $ApoE^{-/-}$ mice model, lectin like ox-LDL receptor-1 (LOX-1) is suggested to be a modulator of PM induced atherosclerosis and plaque rupture [49]. LOX-1 is the major endothelial cell-surface receptor responsible for binding and internalization of ox-LDL, regulating several atherosclerotic plaque growth and destabilization factors [50]. Apparently, only the particle components are responsible for LOX-1 expression rather than gaseous pollutants suggesting that PM up regulates LOX-1 expression in the atherosclerotic plaques and endothelial cells. LDL can be oxidatively modified *in vitro* by ferric ions at low pH, however, it is not exactly known how LDL oxidation *in vivo* occurs [51].

Cellular interactions of PM in progression of atherosclerosis

In the traditional concept of atherosclerosis, hypercholesterolemia, in particular oxLDL, contributes to activation of endothelial cells (ECs) and infiltration and retention of LDL in the intima, inducing inflammation in the vascular wall [13]. In a PM driven atherosclerosis model, one may expect that systemic inflammatory and pro-oxidant effects of PM exacerbate EC

damage [52]. Indeed, exposure to UFPs in mice caused an oxidative stress-driven endothelial dysfunction [53]. Furthermore, in an *in vitro* experimental setup, UFPs directly caused inflammatory effects and inhibition of cell growth in human umbilical vein ECs (HUVECs) [54]. Additionally, increased monocyte chemoattractant protein-1 (MCP-1) and reduced, anti-atherogenic, endothelial nitric oxide (NO) synthase, were observed in healthy male subjects [55]. Exposure to DEPs was related to impairment of vascular endothelial function in $ApoE^{-/-}$ mice [56] and in humans [57] possibly due to decreased NO production [58]. Release of cell adhesion molecules from activated endothelium (such as VCAM-1) mediates leukocytes to roll along the vasculature and finally to adhere at the site of activation [59]. Subsequently, leukocytes and especially monocytes, will migrate through the interendothelial junctions into the subendothelial space, followed by differentiation into macrophages [60]. Ox-LDL will be taken up by macrophages, which will transform into foam cells, the prototypical cell in atherosclerosis [44]. *In vitro*, increased migration of monocytes through HUVECs treated with DEPs has been shown [61]. DEPs also directly caused inflammatory responses in both HUVECs and macrophages. Consistently, monocyte recruitment in atherosclerotic plaques [62] and acceleration of monocyte release from bone marrow [63] after exposure to PM have been demonstrated in atherosclerosis prone watanabe heritable hyperlipidemic (WHHL) rabbits. The activated macrophage produces inflammatory cytokines, and proteases, as well as cytotoxic oxygen and nitrogen radical molecules [13]. Other inflammatory leukocytes such as CD4+ T cells, mast cells and dendritic cells will be involved in this ongoing complex inflammatory process [16]. Concomitantly with macrophages, T-cells infiltrate the atherosclerotic lesions as demonstrated in earlier studies [64]. Several studies with PM, in particular DEPs, showed dendritic cell activation and their promotion of a Th2-like cytokine response by CD4+ T cells, both in humans [65] and in mice [66-68]. Although vascular mast cells are rare, they are found in atherosclerotic plaques especially in the rupture prone shoulder regions [69] and they might destabilize atherosclerotic plaques.

PM induced Atherosclerosis in Animal models

The first study suggesting PM induced atherosclerosis showed that intratracheal administration of PM10 particles in WHHL rabbits increased atherosclerosis by more than 80% as visualized in left main coronary artery (LMCA) and the proximal part of the right coronary artery (RCA), after 4 weeks [12]. The lesions in the left anterior descending coronary artery (LAD) and the left circumflex coronary artery (LCx) were less than 10% and there was no significant difference in atherosclerosis of aorta, when PM exposure was compared to

control. Plaque morphology showed increase in smooth muscle cells, extracellular matrix and large lipids upon PM exposure. Additionally, the particle positive macrophages were correlated with volume fraction of atherosclerotic plaque. Another study group identified PM10 induced lesions in a similar study design with WHHL rabbits but also showed accelerated release of monocytes from bone marrow and phagocytosed particles on the surface of alveolar macrophages [63]. These acute as well as long term effects of PM10 in WHHL rabbits were investigated to give a better understanding of PM induced atherosclerosis [62]. It is suggested that expression of CD31 (platelet endothelial cell adhesion molecule; PECAM-1) and CD49d (which binds to VCAM-1 and stabilizes adhesion of lymphocytes to endothelial cells) on monocytes were increased at 2 hours after PM10 administration. However, repeated exposure to PM10 decreased expression of the monocyte activation markers CD18/CD11b on circulating monocytes, suggesting a removal of activated monocytes, possibly in the lung. Consistently, enhanced ICAM-1 and VCAM-1 expression on the endothelium covering atherosclerotic plaques as well as intra plaque tissue has been shown in rabbits exposed to PM10 [62]. These adhesion molecules are known to promote monocyte adhesion to endothelium and migration into atherosclerotic plaques [70]. Detailed analysis of PM10 induced atherosclerotic plaques in WHHL rabbits with electron microscopy and with immunohistochemistry suggested a vulnerable plaque phenotype characterized by increased migration of macrophage derived foam cells to just below the endothelium, while these were located in core regions of the plaques in controls [71]. Consistent with these studies in WHHL rabbits, PM induced atherosclerosis has also been shown in atherogenic $ApoE^{-/-}$ mice. Exposure to concentrated PM2.5 particles increased atherosclerosis in the abdominal aorta and aortic arch of $ApoE^{-/-}$mice [11, 72, 73] after 6 months. Additionally, increased tissue factor expression and macrophage numbers were observed within the plaque areas of PM2.5 exposed mice and lesions were especially larger in mice fed a high fat diet. Under similar experimental conditions, $ApoE^{-/-}$ mice fed a normal chow diet developed thicker atherosclerotic plaques after UFPs than after PM2.5 challenge, whereas the same abundance of macrophages and SMCs were observed [74]. The relatively more proatherogenic effects of UFPs were explained by more pro-oxidative and ROS production potential of UFPs compared to PM2.5 [75]. Additionally, plasma high density lipoprotein (HDL) of UFPs exposed mice was less anti-inflammatory than PM2.5 exposed mice. A recent study comparing cigarette smoking (4 and 6 months) and PM2.5 (3 and 6 months) exposure in $ApoE^{-/-}$ mice, suggested greatest atherosclerotic plaque formation in LMCA of mice after cigarette smoking, whereas enhanced plaque areas were observed in both brachiocephalic and LMCA of PM2.5 exposed mice [76].

Although the mass concentration of PM2.5 is 30 % less than cigarette smoke, comparable effects were seen in the majority of atherosclerotic lesions. Finally, changes in atherosclerotic plaque morphology after DEPs inhalation in $ApoE^{-/-}$ mice have been documented in detail [5]. Although there was no difference in the volume fraction of plaques between filtered air and DEPs exposure group, histological characterization of plaque morphology revealed increased plaque cellularity by 1.5 to 3 fold after DEPs exposure proposed with increased foam cell formation, lipid accumulation and smooth muscle cell content. The expression of oxidative stress markers, inducible nitric oxide synthase (iNOS), CD36, and nitrotyrosine was significantly increased by 1.5 to 2-fold in plaques, with enhanced systemic lipid and DNA oxidation. In the meantime, increased foam cells and the expression of iNOS and CD36 in plaques were positively correlated with the magnitude of DEPs exposure. Thereby, exposure to DEPs may promote unstable vulnerable plaque characteristics by increased oxidative stress and lipid modulation.

Human atherosclerosis studies with PM

An epidemiological study first hinted to an association between atherosclerosis and ambient air pollution in inhabitants of Los Angeles [9]. When intima-media thickness (CIMT) in the right common carotid artery was investigated as an indicator of future clinical cardiovascular events, 4.4% increase in CIMT was associated with 10 $\mu g/m^3$ increase of PM2.5 particles in ambient air after adjustment for age, sex, education, and income. Although this cross-sectional association was confirmed in another cohort study, these investigators did not show any association between PM (PM10 and PM2.5) and subclinical atherosclerotic disease (early stages of atherosclerosis in peripheral but not in coronary arteries) during follow up, using CIMT, coronary artery calcification (CAC) and ankle-brachial index (ABI) measurements [6]. In contrast, a residential exposure study showed that PM2.5 exposure (3.91 $\mu g/m^3$ increase) was associated with a 17.2% increase in CAC as an early predictor of stenosis [7]. CAC association was more consistent with traffic exposure than with PM2.5. Individuals living more than 200 meters away from a major road had lower CAC risk than persons living closer than 200 meters. Consistent with this finding, ABI as a continuous marker for the degree of subclinical atherosclerosis was positively associated with shortest distance to major roads in a cohort study [77], possibly due to high UFPs concentration since the association with PM2.5 was inconsistent. Another group of investigators also did not find any strong association of PM2.5 or roadway PM with abdominal aortic calcification measured by CT scan however, they did not investigate UFPs [78]. This study may support the hypothesis of a relationship between

PM and systemic atherosclerosis. Furthermore, a cross sectional study for the first time suggested an association between exposure to PM and progression of atherosclerosis measured by CIMT in humans [8]. In support of earlier data, annual CIMT progression among those living within 100 meters of a highway was accelerated. Finally, a positive association between long term exposure to traffic-related particles (PM2.5) and occurrence of acute myocardial infarction has been shown [79]. Traffic exposure was also independently associated with incident coronary artery disease [80].

Although the presented human studies clearly suggested a role for PM in progression of atherosclerosis, more experimental studies are required to better understand the underlying mechanisms behind PM derived atherosclerosis.

Conclusion

In conclusion, there is clear evidence from animal experimental studies that PM affects progression of atherosclerosis at different stages from the initiation phase to advanced atherosclerosis. A mechanistic outlook suggests that pro-oxidative, pro-apoptotic, and pro-inflammatory effects of PM modulate the atherogenic events. In the initial phase, PM generates increased oxidative stress and ultimately triggers the intrinsic pathway of cell apoptosis. PM induced oxidative stress and DNA damage ultimately mediates endothelial dysfunction directly or via systemic inflammation. Endothelial dysfunction (which triggered by and associated with accumulation of LDL) and formation of ox-LDL are the initial events in atherogenesis. Subsequent adhesion and activation of monocytes and migration of T lymphocytes into the subendothelial space aggravates the initiation phase of atherosclerosis. Uptake of ox-LDL by macrophages leads to the formation of foam cells, and from that stage, atherosclerotic plaque formation is accelerated by foam cells via sustaining proinflammatory pathways. Since atherosclerosis is a lifelong and chronic inflammatory disease, the continuous exposure to PM will lead to advanced atherosclerotic plaque formation and cardiovascular complications.

Although long-term exposure to higher levels of ambient PM2.5 in humans might accelerate the progression of atherosclerosis, more investigations are needed. Available data centers around PM2.5, however, the distinct pathways in mediating atherosclerosis specifically by UFPs exposure have not been investigated. Future research is needed to fully elucidate whether UFPs are more harmful to the cardiovascular system or pose a relatively greater proatherogenic than the other types of PM. Time course studies may benefit to our

understanding to reduce risk for cardiovascular effects of PM and define susceptible individuals or vulnerable populations.

References

1 Brook RD, Rajagopalan S, Pope CA, 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC, Jr., Whitsel L, Kaufman JD. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation*. 2010; **121**: 2331-78.

2 Hong YC, Lee JT, Kim H, Ha EH, Schwartz J, Christiani DC. Effects of air pollutants on acute stroke mortality. *Environ Health Perspect*. 2002; **110**: 187-91.

3 Pope CA, 3rd, Renlund DG, Kfoury AG, May HT, Horne BD. Relation of heart failure hospitalization to exposure to fine particulate air pollution. *Am J Cardiol*. 2008; **102**: 1230-4.

4 Rosenlund M, Berglind N, Pershagen G, Hallqvist J, Jonson T, Bellander T. Long-term exposure to urban air pollution and myocardial infarction. *Epidemiology*. 2006; **17**: 383-90.

5 Bai N, Kido T, Suzuki H, Yang G, Kavanagh TJ, Kaufman JD, Rosenfeld ME, van Breemen C, Eeden SF. Changes in atherosclerotic plaques induced by inhalation of diesel exhaust. *Atherosclerosis*. 2011; **216**: 299-306.

6 Diez Roux AV, Auchincloss AH, Franklin TG, Raghunathan T, Barr RG, Kaufman J, Astor B, Keeler J. Long-term exposure to ambient particulate matter and prevalence of subclinical atherosclerosis in the Multi-Ethnic Study of Atherosclerosis. *Am J Epidemiol*. 2008; **167**: 667-75.

7 Hoffmann B, Moebus S, Mohlenkamp S, Stang A, Lehmann N, Dragano N, Schmermund A, Memmesheimer M, Mann K, Erbel R, Jockel KH. Residential exposure to traffic is associated with coronary atherosclerosis. *Circulation*. 2007; **116**: 489-96.

8 Kunzli N, Jerrett M, Garcia-Esteban R, Basagana X, Beckermann B, Gilliland F, Medina M, Peters J, Hodis HN, Mack WJ. Ambient air pollution and the progression of atherosclerosis in adults. *PLoS One*. 2010; **5**: e9096.

9 Kunzli N, Jerrett M, Mack WJ, Beckerman B, LaBree L, Gilliland F, Thomas D, Peters J, Hodis HN. Ambient air pollution and atherosclerosis in Los Angeles. *Environ Health Perspect*. 2005; **113**: 201-6.

10 Quan C, Sun Q, Lippmann M, Chen LC. Comparative effects of inhaled diesel exhaust and ambient fine particles on inflammation, atherosclerosis, and vascular dysfunction. *Inhal Toxicol*. 2010; **22**: 738-53.

11 Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *Jama*. 2005; **294**: 3003-10.

12 Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, van Eeden SF. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol*. 2002; **39**: 935-42.

13 Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005; **352**: 1685-95.

14 Mizuno Y, Jacob RF, Mason RP. Inflammation and the development of atherosclerosis. *J Atheroscler Thromb*. 2011; **18**: 351-8.

15 Hansson GK. Immune and inflammatory mechanisms in the development of atherosclerosis. *Br Heart J*. 1993; **69**: S38-41.

16 Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol*. 2008; **8**: 802-15.

17 Danielsen PH, Moller P, Jensen KA, Sharma AK, Wallin H, Bossi R, Autrup H, Molhave L, Ravanat JL, Briede JJ, de Kok TM, Loft S. Oxidative stress, DNA damage, and inflammation induced by ambient air and wood smoke particulate matter in human A549 and THP-1 cell lines. *Chem Res Toxicol*. 2011; **24**: 168-84.

18 Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J, Nel A. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ Health Perspect*. 2003; **111**: 455-60.

19 Victor VM, Rocha M, Sola E, Banuls C, Garcia-Malpartida K, Hernandez-Mijares A. Oxidative stress, endothelial dysfunction and atherosclerosis. *Curr Pharm Des*. 2009; **15**: 2988-3002.

20 Nakajima K, Nakano T, Tanaka A. The oxidative modification hypothesis of atherosclerosis: the comparison of atherogenic effects on oxidized LDL and remnant lipoproteins in plasma. *Clin Chim Acta*. 2006; **367**: 36-47.

21 Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect*. 2005; **113**: 1555-60.

22 Furuyama A, Kanno S, Kobayashi T, Hirano S. Extrapulmonary translocation of intratracheally instilled fine and ultrafine particles via direct and alveolar macrophage-associated routes. *Arch Toxicol*. 2009; **83**: 429-37.

23 Moller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, Vesterdal LK, Forchhammer L, Wallin H, Loft S. Role of oxidative damage in toxicity of particulates. *Free Radic Res*. 2010; **44**: 1-46.

24 Araujo JA. Particulate air pollution, systemic oxidative stress, inflammation, and atherosclerosis. *Air Qual Atmos Health*. 2010; **4**: 79-93.

25 Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environ Health Perspect*. 2002; **110**: 749-55.

26 Pilger A, Rudiger HW. 8-Hydroxy-2'-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures. *Int Arch Occup Environ Health*. 2006; **80**: 1-15.

27 Valavanidis A, Vlahoyianni T, Fiotakis K. Comparative study of the formation of oxidative damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) adduct from the nucleoside 2'-deoxyguanosine by transition metals and suspensions of particulate matter in relation to metal content and redox reactivity. *Free Radic Res*. 2005; **39**: 1071-81.

28 Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2009; **27**: 120-39.

29 Kim JY, Mukherjee S, Ngo LC, Christiani DC. Urinary 8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to fine particulates. *Environ Health Perspect*. 2004; **112**: 666-71.

30 Ichinose T, Yajima Y, Nagashima M, Takenoshita S, Nagamachi Y, Sagai M. Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis*. 1997; **18**: 185-92.

31 Tokiwa H, Sera N, Nakanishi Y, Sagai M. 8-Hydroxyguanosine formed in human lung tissues and the association with diesel exhaust particles. *Free Radic Biol Med*. 1999; **27**: 1251-8.

32 Coultas L, Strasser A. The molecular control of DNA damage-induced cell death. *Apoptosis*. 2000; **5**: 491-507.

33 Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans*. 2001; **29**: 684-8.

34 Hiura TS, Kaszubowski MP, Li N, Nel AE. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J Immunol*. 1999; **163**: 5582-91.

35 Soberanes S, Panduri V, Mutlu GM, Ghio A, Bundinger GR, Kamp DW. p53 mediates particulate matter-induced alveolar epithelial cell mitochondria-regulated apoptosis. *Am J Respir Crit Care Med*. 2006; **174**: 1229-38.

36 Soberanes S, Urich D, Baker CM, Burgess Z, Chiarella SE, Bell EL, Ghio AJ, De Vizcaya-Ruiz A, Liu J, Ridge KM, Kamp DW, Chandel NS, Schumacker PT, Mutlu GM, Budinger GR. Mitochondrial complex III-generated oxidants activate ASK1 and JNK to induce alveolar epithelial cell death following exposure to particulate matter air pollution. *J Biol Chem*. 2009; **284**: 2176-86.

37 Urich D, Soberanes S, Burgess Z, Chiarella SE, Ghio AJ, Ridge KM, Kamp DW, Chandel NS, Mutlu GM, Budinger GR. Proapoptotic Noxa is required for particulate matter-induced cell death and lung inflammation. *Faseb J*. 2009; **23**: 2055-64.

38 Naik E, Michalak EM, Villunger A, Adams JM, Strasser A. Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. *J Cell Biol*. 2007; **176**: 415-24.

39 Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res*. 2000; **45**: 736-46.

40 Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci (Lond)*. 2004; **107**: 343-54.

41 Bennett MR. Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res*. 1999; **41**: 361-8.

42 Leskinen MJ, Kovanen PT, Lindstedt KA. Regulation of smooth muscle cell growth, function and death in vitro by activated mast cells--a potential mechanism for the weakening and rupture of atherosclerotic plaques. *Biochem Pharmacol*. 2003; **66**: 1493-8.

43 Kaplan M, Aviram M. Oxidized low density lipoprotein: atherogenic and proinflammatory characteristics during macrophage foam cell formation. An inhibitory role for nutritional antioxidants and serum paraoxonase. *Clin Chem Lab Med*. 1999; **37**: 777-87.

44 Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. *Rev Esp Cardiol*. 2009; **62**: 1161-78.

45 Meisel SR, Xu XP, Edgington TS, Dimayuga P, Kaul S, Lee S, Fishbein MC, Cercek B, Shah PK. Differentiation of adherent human monocytes into macrophages markedly enhances tissue factor protein expression and procoagulant activity. *Atherosclerosis*. 2002; **161**: 35-43.

46 Kita T, Kume N, Minami M, Hayashida K, Murayama T, Sano H, Moriwaki H, Kataoka H, Nishi E, Horiuchi H, Arai H, Yokode M. Role of oxidized LDL in atherosclerosis. *Ann N Y Acad Sci*. 2001; **947**: 199-205; discussion -6.

47 Jacobs L, Emmerechts J, Hoylaerts MF, Mathieu C, Hoet PH, Nemery B, Nawrot TS. Traffic air pollution and oxidized LDL. *PLoS One*. 2011; **6**: e16200.

48 Soares SR, Carvalho-Oliveira R, Ramos-Sanchez E, Catanozi S, da Silva LF, Mauad T, Gidlund M, Goto H, Garcia ML. Air pollution and antibodies against modified lipoproteins are associated with atherosclerosis and vascular remodeling in hyperlipemic mice. *Atherosclerosis*. 2009; **207**: 368-73.

49 Lund AK, Lucero J, Harman M, Madden MC, McDonald JD, Seagrave JC, Campen MJ. The oxidized low-density lipoprotein receptor mediates vascular effects of inhaled vehicle emissions. *Am J Respir Crit Care Med*. 2011; **184**: 82-91.

50 Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. An endothelial receptor for oxidized low-density lipoprotein. *Nature*. 1997; **386**: 73-7.

51 Yoshida H, Kisugi R. Mechanisms of LDL oxidation. *Clin Chim Acta*. 2010; **411**: 1875-82.

52 Zhu MT, Wang B, Wang Y, Yuan L, Wang HJ, Wang M, Ouyang H, Chai ZF, Feng WY, Zhao YL. Endothelial dysfunction and inflammation induced by iron oxide nanoparticle exposure: Risk factors for early atherosclerosis. *Toxicol Lett*. 2011; **203**: 162-71.

53 Mo Y, Wan R, Chien S, Tollerud DJ, Zhang Q. Activation of endothelial cells after exposure to ambient ultrafine particles: the role of NADPH oxidase. *Toxicol Appl Pharmacol*. 2009; **236**: 183-93.

54 Yamawaki H, Iwai N. Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. *Circ J*. 2006; **70**: 129-40.

55 Briet M, Collin C, Laurent S, Tan A, Azizi M, Agharazii M, Jeunemaitre X, Alhenc-Gelas F, Boutouyrie P. Endothelial function and chronic exposure to air pollution in normal male subjects. *Hypertension*. 2007; **50**: 970-6.

56 Hansen CS, Sheykhzade M, Moller P, Folkmann JK, Amtorp O, Jonassen T, Loft S. Diesel exhaust particles induce endothelial dysfunction in apoE-/- mice. *Toxicol Appl Pharmacol*. 2007; **219**: 24-32.

57 Tornqvist H, Mills NL, Gonzalez M, Miller MR, Robinson SD, Megson IL, Macnee W, Donaldson K, Soderberg S, Newby DE, Sandstrom T, Blomberg A. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *Am J Respir Crit Care Med*. 2007; **176**: 395-400.

58 Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004; **109**: III27-32.

59 Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. *Atherosclerosis*. 2003; **170**: 191-203.

60 Engelhardt B, Wolburg H. Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol*. 2004; **34**: 2955-63.

61 Shaw CA, Robertson S, Miller MR, Duffin R, Tabor CM, Donaldson K, Newby DE, Hadoke PW. Diesel exhaust particulate--exposed macrophages cause marked endothelial cell activation. *Am J Respir Cell Mol Biol*. 2011; **44**: 840-51.

62 Yatera K, Hsieh J, Hogg JC, Tranfield E, Suzuki H, Shih CH, Behzad AR, Vincent R, van Eeden SF. Particulate matter air pollution exposure promotes recruitment of monocytes into atherosclerotic plaques. *Am J Physiol Heart Circ Physiol*. 2008; **294**: H944-53.

63 Goto Y, Hogg JC, Shih CH, Ishii H, Vincent R, van Eeden SF. Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits. *Am J Physiol Lung Cell Mol Physiol*. 2004; **287**: L79-85.

64 Zhou X. CD4+ T cells in atherosclerosis. *Biomed Pharmacother*. 2003; **57**: 287-91.

65 Porter M, Karp M, Killedar S, Bauer SM, Guo J, Williams D, Breysse P, Georas SN, Williams MA. Diesel-enriched particulate matter functionally activates human dendritic cells. *Am J Respir Cell Mol Biol*. 2007; **37**: 706-19.

66 Bezemer GF, Bauer SM, Oberdorster G, Breysse PN, Pieters RH, Georas SN, Williams MA. Activation of pulmonary dendritic cells and Th2-type inflammatory responses on instillation of engineered, environmental diesel emission source or ambient air pollutant particles in vivo. *J Innate Immun*. 2011; **3**: 150-66.

67 Braun A, Bewersdorff M, Lintelmann J, Matuschek G, Jakob T, Gottlicher M, Schober W, Buters JT, Behrendt H, Mempel M. Differential impact of diesel particle composition on pro-allergic dendritic cell function. *Toxicol Sci*. 2010; **113**: 85-94.

68 Williams MA, Rangasamy T, Bauer SM, Killedar S, Karp M, Kensler TW, Yamamoto M, Breysse P, Biswal S, Georas SN. Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. *J Immunol*. 2008; **181**: 4545-59.

69 Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol*. 2009; **27**: 165-97.

70 Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007; **27**: 2292-301.

71 Tranfield EM, van Eeden SF, Yatera K, Hogg JC, Walker DC. Ultrastructural changes in atherosclerotic plaques following the instillation of airborne particulate matter into the lungs of rabbits. *Can J Cardiol*. 2010; **26**: e258-69.

72 Chen LC, Nadziejko C. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. V. CAPs exacerbate aortic plaque development in hyperlipidemic mice. *Inhal Toxicol*. 2005; **17**: 217-24.

73 Sun Q, Yue P, Kirk RI, Wang A, Moatti D, Jin X, Lu B, Schecter AD, Lippmann M, Gordon T, Chen LC, Rajagopalan S. Ambient air particulate matter exposure and tissue factor expression in atherosclerosis. *Inhal Toxicol*. 2008; **20**: 127-37.

74 Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ, Nel AE. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circ Res*. 2008; **102**: 589-96.

75 Jeng HA. Chemical composition of ambient particulate matter and redox activity. *Environ Monit Assess*. 2010; **169**: 597-606.

76 Chen LC, Quan C, Hwang JS, Jin X, Li Q, Zhong M, Rajagopalan S, Sun Q. Atherosclerosis lesion progression during inhalation exposure to environmental tobacco smoke: a comparison to concentrated ambient air fine particles exposure. *Inhal Toxicol*. 2010; **22**: 449-59.

77 Hoffmann B, Moebus S, Kroger K, Stang A, Mohlenkamp S, Dragano N, Schmermund A, Memmesheimer M, Erbel R, Jockel KH. Residential exposure to urban air pollution, ankle-brachial index, and peripheral arterial disease. *Epidemiology*. 2009; **20**: 280-8.

78 Allen RW, Criqui MH, Diez Roux AV, Allison M, Shea S, Detrano R, Sheppard L, Wong ND, Stukovsky KH, Kaufman JD. Fine particulate matter air pollution, proximity to traffic, and aortic atherosclerosis. *Epidemiology*. 2009; **20**: 254-64.

79 Tonne C, Yanosky J, Gryparis A, Melly S, Mittleman M, Goldberg R, von Klot S, Schwartz J. Traffic particles and occurrence of acute myocardial infarction: a case-control analysis. *Occup Environ Med*. 2009; **66**: 797-804.

80 Kan H, Heiss G, Rose KM, Whitsel EA, Lurmann F, London SJ. Prospective analysis of traffic exposure as a risk factor for incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *Environ Health Perspect*. 2008; **116**: 1463-8.

OUTLINE OF THIS THESIS

Air pollution is a part of our daily urban life, containing particulate matter (PM) in different sizes and gases. Recent observations have implicated that air pollution is associated with thrombosis and atherosclerosis but the underlying mechanism is not fully understood. A mechanistic look on air pollution associated cardiovascular diseases has revealed two distinct pathways: an indirect way, via pro-inflammatory effects (systemic and in lung), and/or a direct way via translocation of small particles and gases into the blood circulation. In both scenarios, a disturbed balance of hemostasis is the main contributor to thrombosis.

In this thesis, I report possible procoagulant effects of traffic related particulate matter and gases based on these two distinct pathways in extensive experimental studies, *in vitro*, as well as in animals and humans. The focus of our research concentrated on the two main pathways of blood coagulation; the tissue factor dependent extrinsic pathway and especially the factor XII dependent intrinsic pathway. From the cardiovascular perspective, the effects of diesel and biodiesel particles on the progression of atherosclerosis are also presented with a detailed analysis of morphology and composition of plaques.

In **chapter 1- part I**, the recent literature regarding the effects of particulate matter on coagulation, hemostasis and thrombosis is summarized. Particulate matter is considered according to the aerodynamic diameters and, based on the key findings of human and animal studies, mechanistic hypotheses are suggested. Furthermore, as a main cause of cardiovascular disease, the effects of PM exposure on atherosclerosis are discussed in **chapter 1-part II**. The effects of PM on characteristic cell constituents of atherosclerotic lesions and several pathophysiologic conditions involved in atherosclerosis, such as oxidative stress, apoptosis, oxidized LDL, are being addressed. In **chapter 2,** the results of a population study in 40 healthy subjects exposed to PM10 (PM10 <10 μm) and corresponding gas pollutants (CO, NO, NO_2 and O_3) and the changes in platelet aggregation, inflammation and thrombin generation parameters (tissue factor dependent) are presented. In **chapter 3**, changes in vascular function and tissue factor dependent blood coagulation measured by thrombin generation in extent to pulmonary and cardiovascular effects of traffic related PM2.5 (PM2.5 <2.5μm) and diesel exhaust in rats, are discussed. **Chapter 4** deals with the procoagulant effects of PM of different sizes, *in vitro* and particularly of ultrafine particles, in mice. We investigated the effects of PM on the intrinsic pathway of coagulation and for the first time were able to establish a role for factor XII in modulating its procoagulant effects. Furthermore, in a randomized study, we exposed healthy volunteers to diesel exhaust and measured thrombin generation at different time points after exposure. The outcome of this study is described in **chapter 5**. In **chapter 6,** we report the outcome of exposure to diesel or biodiesel particles on the phenotype of atherosclerosis in LDL -/- mice. Numbers of immunohistochemical analysis were performed to determine the morphology and composition of atherosclerotic plaques. Finally, the findings of these studies are discussed in **chapter 7**.

CHAPTER 2

EFFECTS OF AMBIENT AIR POLLUTION ON HEMOSTASIS AND INFLAMMATION

Based on:

Rudež, G., Janssen, N.A.H., Kilinc, E., Leebeek, F.W.G., Gerlofs-Nijland, M.E., Spronk, H.M.H., ten Cate, H., Cassee F.R., de Maat, M.P.M.

2009. Effects of ambient air pollution on hemostasis and inflammation. *Environ. Health Perspect.* 117(6):995-1001.

Abstract

Air pollution has consistently been associated with increased morbidity and mortality due to respiratory and cardiovascular disease. Underlying biological mechanisms are not entirely clear, and hemostasis and inflammation are suggested to be involved.

Our aim was to study the association of the variation in local concentrations of airborne particulate matter (PM) with aerodynamic diameter < 10 μm, carbon monoxide, nitrogen monoxide, nitrogen dioxide, and ozone with platelet aggregation, thrombin generation, fibrinogen, and C-reactive protein (CRP) levels in healthy individuals.

From 40 healthy volunteers, we collected 13 consecutive blood samples within a 1-year period and measured light-transmittance platelet aggregometry, thrombin generation, fibrinogen, and CRP. We performed regression analysis using generalized additive models to study the association between the hemostatic and inflammatory variables, and local environmental concentrations of air pollutants for time lags within 24 h before blood sampling or 24–96 h before blood sampling.

In general, air pollutants were associated with platelet aggregation [average, +8% per interquartile range (IQR), $p < 0.01$] and thrombin generation (average, +1% per IQR, $p < 0.05$). Platelet aggregation was not affected by *in vitro* incubation of plasma with PM. We observed no relationship between any of the air pollutants and fibrinogen or CRP levels.

Air pollution increased platelet aggregation as well as coagulation activity but had no clear effect on systemic inflammation. These prothrombotic effects may partly explain the relationship between air pollution and the risk of ischemic cardiovascular disease.

Introduction

Epidemiologic studies have linked elevated levels of both gaseous and (ultra-)fine particulate matter (PM) ambient air pollutants to increased morbidity and mortality due to respiratory and cardiovascular disease [1, 2]. Underlying biological mechanisms are unclear, but inflammation and hemostasis are suggested to be involved [3-5]. It has been postulated that inhaled gases, and also ultrafine PM because of its very small particle size (< 0.1 μm), can readily cross the lung epithelium into the bloodstream [6]. There they can have direct, transient systemic effects leading to a prothrombotic state, such as enhanced platelet activation and thrombin generation [7-9]. In contrast, larger particles that cannot pass the alveolar–blood barrier will perturb the lung epithelium, where they may give rise to local inflammation [10, 11]. Under experimental conditions, human or animal exposure to a controlled high dose of air pollutants has been shown to cause pulmonary inflammation that leads to a systemic release of cytokines. This in turn induces *de novo* synthesis of inflammatory biomarkers in the liver, such as fibrinogen, which also plays a major part in blood clotting, and C-reactive protein (CRP). In general, at least 24 h elapses from the onset of this protein synthesis to a clear increase in plasma levels of inflammatory markers [5]. We therefore hypothesized that air pollution has both direct and indirect effects on platelet aggregation and coagulation, but only indirect effects on plasma levels of the inflammatory variables fibrinogen and CRP.

Previous studies have aimed primarily at finding epidemiologic associations between concentrations of air pollution and health effects, including mortality, or associations between experimentally controlled exposures to air pollution and various biological variables in human and animal models [12, 13]. However, studies are still lacking that focus on the effect of air pollution on hemostasis and inflammation in a real-life urban situation over a longer period of time, especially because continuous long-term monitoring shows large variations within each year in local concentrations of air pollutants (Fig. 2.1) [3]. Therefore, the aim of this study was to investigate longitudinally (i.e., repeatedly over 1 year) the associations between local urban concentrations of ambient air pollution and plasma markers of hemostasis and inflammation. In addition, we aimed to investigate *in vitro* whether PM can have an effect on platelet aggregation.

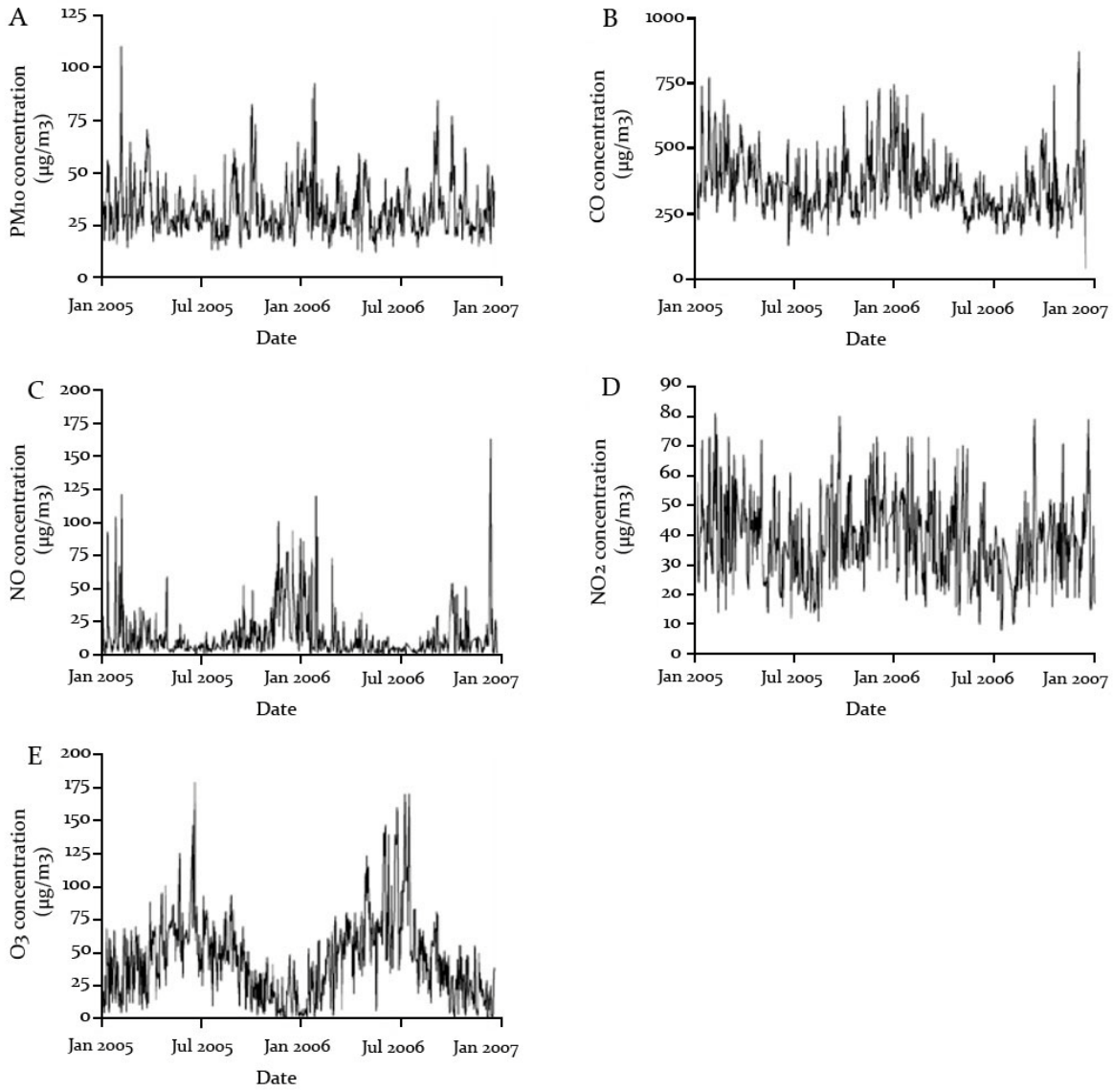


Figure 2.1. ***Concentration profiles of air pollutants during the study period***
24 h mean concentrations for PM10 (A), CO (C), and NO_2 (D) and 8 h mean concentrations (1200 to 2000 hours) for O_3 (E).

Materials and methods

Study population

Between January 2005 and December 2006, we included 40 healthy individuals who were living or working in the city center of Rotterdam, the Netherlands, a city agglomerate with almost 1 million inhabitants. Exclusion criteria were symptoms of chronic infectious diseases, acute infections, or any surgical procedure within the preceding 3 months. We collected from each participant blood at 11–13 (mean, 12.5) different visits throughout a 1-year period. In total, we collected 498 blood samples on 197 days. For each subject, the minimal interval between successive blood collections was 3 days and the maximal interval was 6 months, with a similar pattern of distribution in each subject. We collected data on demographics and cardiovascular risk factors using a standardized questionnaire. To minimize the effect of circadian variation on plasma levels of biomarkers, we took blood samples between 09^{00} and 11^{00} hours. The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Erasmus University Medical Center. We obtained written informed consent from each participant.

Air pollution monitoring data

We obtained concentrations of PM with aerodynamic diameter < 10 μm (PM10), carbon monoxide, nitrogen monoxide, nitrogen dioxide, and ozone from the Dutch National Air Quality Monitoring Network (National Institute for Public Health and the Environment 2009[14]), which measured these air pollutants hourly at monitoring station no. 418 (Schiedamse Vest, Rotterdam, the Netherlands). This monitoring site is located in the Rotterdam city center and is subject to frequent quality control to ensure its ability to represent urban background air pollution. For data analysis, we calculated 6-h means and then combined them into 12-h and 24-h means. If more than two hourly concentrations were missing for a 6-h mean, we imputed them using data from five other monitoring stations of the Dutch National Air Quality Monitoring Network that were all within 25 km of Rotterdam.

Blood collection

Blood was drawn by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration, 3.2%). Plasma was

obtained by centrifugation at 1,500*g* for 10 min at 4°C and stored in aliquots at –80°C until further analysis. For platelet aggregation, blood was centrifuged at 150*g* for 15 min to obtain platelet-rich plasma (PRP) and subsequently at 1,500*g* for 10 min to obtain platelet-poor plasma (PPP). We adjusted PRP with autologous PPP to 200 × 109 platelets/L (P200), which was used in platelet aggregation experiments.

Laboratory measurements

Light-transmittance platelet aggregometry

We performed adenosine diphosphate (ADP) induced light transmittance platelet aggregometry as described previously [15]. We chose ADP as the agonist because the ADP pathway in platelets plays an important role in atherothrombosis [16]. We preincubated P200 with aspirin (100 μmol/L final) for 20 min and brought it to a physiologic calcium concentration of 16.6 mM by adding calcium chloride ($CaCl_2$) (Merck & Co., New York, NY, USA) after preincubation with the thrombin inhibitor d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (40 μmol/L, final; Merck & Co., Darmstadt, Germany). We induced platelet aggregation by 5 and 2.5 μmol/L ADP (Sigma Chemical Co., St. Louis, MO, USA) and determined maximal aggregation and late aggregation (residual aggregation at 6 min after the maximum representing platelet aggregate stability) by recording for 10 min on a four channel optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, the Netherlands). Because of logistic reasons, platelet aggregation could be performed in only a subset of 139 plasma samples from 16 individuals.

We studied direct *in vitro* effects of PM on platelet aggregation by adding different types of diluted PM (reference PM with diameter size < 0.1, 2.5, or 10 μm, diesel soot collected with a diesel generator, urban background dust collected from a local baghouse filter extract, or EHC-93 reference dust that was collected in Ottawa, Ontario, Canada) to P200 or whole blood in various concentrations (range, 0–100 μg/mL) for different incubation periods (range, 0–2 h) and performing ADP induced light transmittance or ADP induced impedance whole blood platelet aggregation experiments (Chrono-log), respectively. We compared results with those obtained with aliquot samples without incubation with PM.

Thrombin generation

We measured thrombin generation in tissue factor (TF) triggered PPP with the calibrated automated thrombogram (CAT) method (Thrombinoscope, Maastricht, the Netherlands) [17]. We conducted measurements on 80 μL plasma with final concentrations of 1 and 5 pM TF

(PPP reagent low and PPP reagent; Thrombinoscope) and 4 µM phospholipids. We obtained thrombin calibrator from Thrombinoscope. We read fluorescence in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 nm filter set. We calculated thrombin generation curves with the Thrombinoscope software. We derived three parameters from the thrombin generation curves: lag time (defined as the time to reach one-sixth of the peak height), endogenous thrombin potential (ETP), and peak height. A thrombin generation curve is characterized by the initial burst of thrombin formation and the lag time, which depends on the amount of TF present in the sample or added to the plasma to trigger coagulation. Furthermore, the lag time is negatively associated with the plasma levels of factors VII and IX, antithrombin, free protein S, and free TF pathway inhibitor [18]. The other two main parameters, ETP and peak height, reflect the potential of plasma to generate thrombin and have been suggested to indicate a state of hypercoagulability when elevated [19]. Both the ETP and peak height are determined by plasma levels of fibrinogen, factor XII, antithrombin, and free TF pathway inhibitor [18].

Fibrinogen and CRP

We determined fibrinogen levels according to von Clauss (Instrumentation Laboratory, IJsselstein, the Netherlands) and the prothrombin (PT) derived method (Dade Thrombin Reagent, Siemens Diagnostics, Leusden, the Netherlands) on a Sysmex CA-1500 automated coagulation analyzer (Siemens Diagnostics, Leusden, the Netherlands). We measured CRP levels by means of an in house high sensitivity ELISA with polyclonal rat anti human CRP antibodies (Dako, Glostrup, Denmark) and a CRP calibrator (Dako).

Statistical analysis

We present data as mean ± SD for continuous variables and as counts and percentages for categorical variables. We performed linear regression analysis between plasma levels and air pollution concentration at different periods before each blood sampling. We analyzed data in R software (version 2.5.1; R Foundation for Statistical Computing, Vienna, Austria) using generalized additive models with individual intercepts for each subject, day of the week as a an indicator variable, and penalized spline smoothers for date (to adjust for trend and seasonality) and meteorologic parameters (temperature, pressure, and relative humidity). We used the software to optimize degrees of freedom used for the splines, according to the procedure described by [20]. We used 10 knots as a starting point. The effective

degrees of freedom for trend ranged from 1 to 8 for the different models. A time lag corresponds to a mean concentration of an air pollutant that we calculated from concentrations hourly measured within the corresponding time window preceding each blood sampling, for which we set the time of the blood sampling to 0 hours. Time lags represent direct effects D0–6, D0–12, and D0–24; indirect effects I24–48, I48–72, and I72–96; and both direct and indirect effects D+I0–96 (Fig. 2.2). In addition, for O_3 we added the maximum concentration that we measured within the 24 h preceding each blood sampling to study the effect of peak exposures. The longitudinal study design included repeated measures analysis, whereby subjects served as their own references. In this analysis, plasma levels in 13 blood samples of each subject were associated with the corresponding local concentrations of air pollutants. We normalized effects of air pollution on plasma variables and present them as percent change of the variable of interest for one interquartile range (IQR) of an air pollutant (%/IQR) (+ indicates an increase of the %/IQR; indicates a decrease). In this model, effects can be compared among all air pollutants and all plasma variables. We considered a two sided value of $p < 0.05$ statistically significant. For CRP and fibrinogen, we determined only indirect effects of air pollution (time lags I24–48, I48–72, and I72–96) [21]. We also performed all analyses after excluding smokers (n = 7) or women using oral contraceptives (n = 9). We calculated the correlation coefficients between the concentrations of different air pollutants by means of Pearson's correlation test.

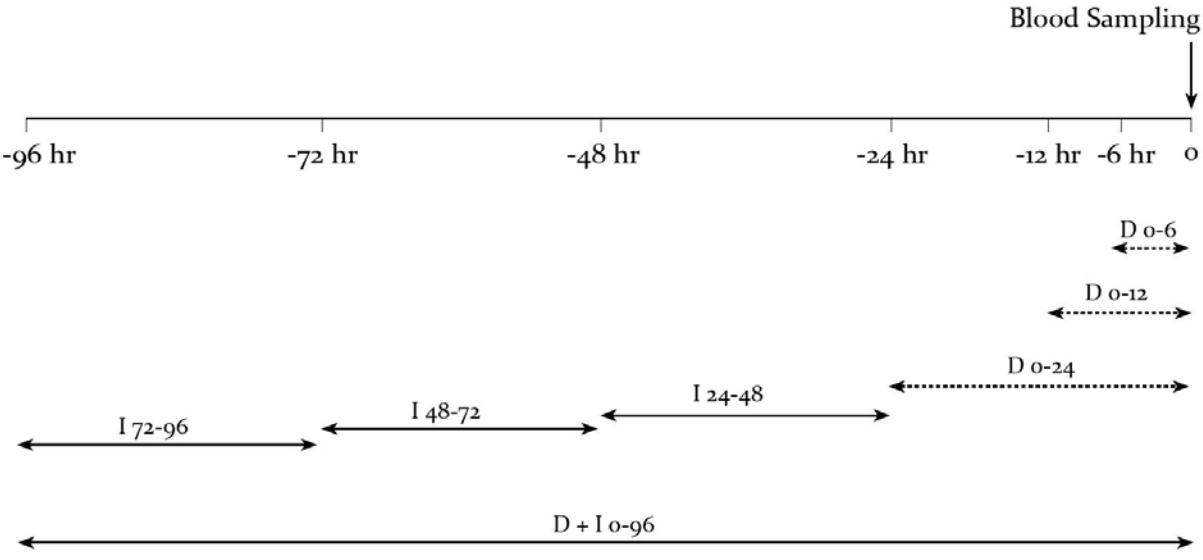


Figure 2. 2. ***Time lags of estimated exposure to air pollution before blood sampling***
The time of each blood sampling was set to 0 hours. Time lags represent means of air pollution concentrations that were determined hourly within the corresponding time window preceding each blood sampling. Dashed arrows represent direct effects of air pollution (D0–6, D0–12, and D0–24), and solid arrows indirect effects (I24–48, I48–72, and I72–96). Time lag D+I0–96 represents the mean concentration within 4 days before blood sampling.

Results

Study population and concentration profiles of air pollutants

We included 40 healthy subjects with a mean age of 41 years in the study (Table 2.1). Twenty six women participated in the study (65% of total). In total, there were seven current smokers (18%).

Table 2.1. *Characteristics of the study population*

Characteristic	**(n=40)**
Age (years)	41 ± 15
No. of females	26 (65%)
Body mass index (kg/m2)	22.6 ± 2.0
No. of smokers	7 (18%)
No. of oral contraceptive users	9 (23%)
Blood parameters	
Fibrinogen (g/L)	2.6 ± 0.5
CRP (mg/L)	0.6 ± 1.2
Platelet aggregation (n=16)	
Maximal aggregation (%)	65 ± 13
Late aggregation (%)	46 ± 20
Thrombin generation	
ETP (nM/min)	999 ± 317
Peak (nM)	141 ± 71
Lag time (min)	4.2 ± 0.9

Values are no. (%) for categorical variables and mean ± SD for continuous variables.

The profile of air pollution concentrations throughout the study period shows quite variable levels of air pollutants (Table 2.2, Fig. 2.1). The correlation coefficients between the concentrations of different air pollutants were > 0.6 per each of the studied time lags and were negative between O_3 and the other air pollutants (0.4 to 0.6).

Table 2.2. *Concentrations (μg/m3) of air pollutants during the study period*

Air Pollutant	**Median**	**25^{th}-75^{th} Percentile**	**Maximum**
PM_{10}	29.3	23.8–39.2	110.1
CO	333	276–412	1,283
NO	7	4–15	163
NO_2	37	27–48	81
O_3	44	21–63	180

Data are presented as $\mu g/m^3$. For each air pollutant, median 25^{th} to 75^{th} percentile and maximal concentration are given.

Platelet aggregation

The characteristics of the subset of subjects in whom platelet aggregation was performed (n = 16) were similar to those of the remaining 24 subjects, except that there were no users of oral contraceptives in this subset of 16 subjects (data not shown). We observed indirect effects of air pollution on platelet aggregation, represented by a positive significant association between 5 µmol/L ADP induced maximal aggregation and PM10 concentrations for time lag I72–96 (+8%/IQR, $p < 0.01$) (Fig. 2.3A).

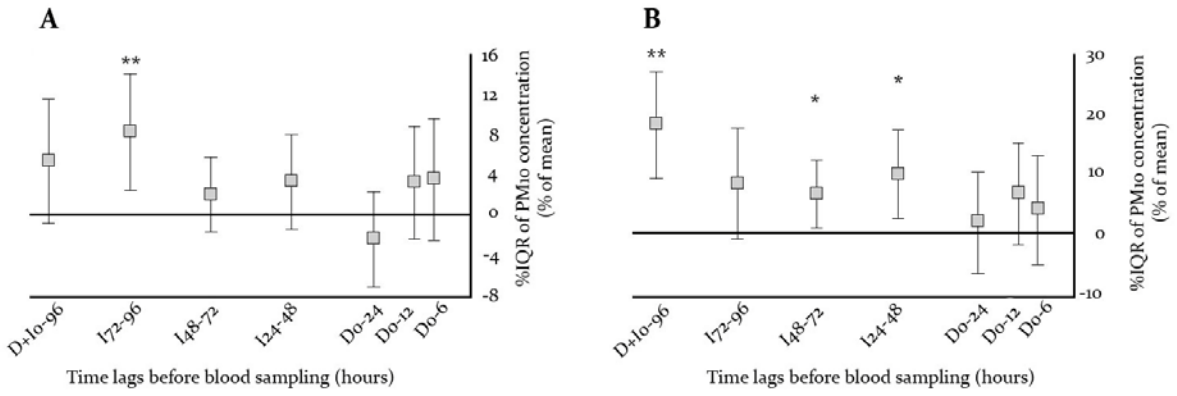


Figure 2.3. ***Effects of PM10 on platelet aggregation***
Estimated effects of PM10 on maximal aggregation (*A*) and late aggregation (*B*) are given as percent change from the mean of each individual per increase in interquartile of PM10 concentration. Time lags D0–6, D0–12, and D0–24 represent direct effects of PM10 on platelet aggregation, and time lags I24–48, I48–72, and I72–96, indirect effects. Time lag D+I0–96 represents the effect of 4-day mean concentration of PM10 on platelet aggregation. *$p < 0.05$, **$p < 0.01$.

In addition, late aggregation was significantly associated with PM10 for time lags I24–48 and I48–72 (+10 and +6%/IQR, respectively; both $p < 0.05$) and for time lag D+I0–96 (+18%/IQR, $p < 0.01$) (Fig. 2. 3B). We also observed significant associations between maximal aggregation and CO, NO, and NO2 for time lag I48–72 (+8, +6, and +6%/IQR, respectively; all $p < 0.01$) and for time lag D+I0–96 (+9%/IQR of CO, $p < 0.05$, and +8%/IQR of NO, $p < 0.01$) (Table 2.3). Similarly, late aggregation was significantly associated with CO, NO, and NO2 for these time lags (I48–72: +18, +8, and +9%/IQR, respectively; D+I0–96: +20, +13, and +16 %/IQR, respectively; all $p < 0.01$) (Table 2. 3). In addition, we observed a significant association between O3 during time lag I48–72 and maximal daily concentration of O3 and late aggregation (–26 and –16%/IQR, respectively, both $p < 0.05$) (Table 2.3). We obtained similar results when we induced platelet aggregation with 2.5 µmol/L ADP, instead of 5 µmol/L ADP, and when we performed the analyses in nonsmokers only or after exclusion of women using oral contraceptives.

No direct effect of PM10 on platelet aggregation was observed, because no associations between the PM10 concentration and maximum platelet aggregation or late aggregation for the direct-effect time lags D0–6, D0–12, and D0–24 were noted (Figure 3). We confirmed this absence of direct effects *in vitro*, because the addition of various types of PM to P200 or whole blood did not lead to any changes in light transmittance or impedance whole blood platelet aggregation, compared with an aliquot P200 sample to which we added no PM. However, a direct *in vivo* effect was suggested for CO, because we observed a significant positive association with late aggregation (+11, +12, and +11%/IQR for D0–6, D0–12, and D0–24, respectively; all $p < 0.05$) (Table 2.3).

Table 2.3. *Estimated changes of platelet aggregation parameters associated with mean air pollutant levels at various time lags preceding blood samplings*

	Air pollutant				
Time lag	PM10	CO	NO	NO_2	O_3
Maximal platelet aggregation					
D0–6	3.5 (−2.5 , 9.6)	−3.6 (−9.3 , 2.1)	1.3 (−4.4 , 7.1)	−2.3 (−7.3 , 2.7)	7.0 (−1.7 , 15.7)
D0–12	3.2 (−2.4 , 8.8)	−4.7 (−11.0 , 1.5)	0.7 (−5.4 , 6.8)	−2.6 (−8.4 , 3.3)	4.1 (−4.6 , 12.8)
D0–24	−2.5 (−7.2 , 2.3)	−2.6 (−7.9 , 2.7)	1.9 (−3.0 , 6.9)	−3.0 (−10.3 , 4.3)	4.9 (−6.6 , 16.3)
I24–48	3.3 (−1.5 , 8.1)	−1.1 (−7.2 , 4.9)	1.2 (−4.1 , 6.5)	−0.6 (−6.6 , 5.3)	−5.7 (−20.3 , 9.0)
I48–72	2.0 (−1.7 , 5.8)	8.4 (2.5 , 14.3)**	6.1 (2.4 , 9.7)**	5.6 (1.5 , 9.7)**	−8.1 (−18.8 , 2.7)
I72–96	8.3 (2.5 , 14.1)**	−0.1 (−5.1 , 5.0)	−0.4 (−5.5 , 4.8)	1.2 (−4.5 , 6.9)	−1.6 (−10.4 , 7.3)
D+I0–96	5.4 (−0.8 , 11.6)	9.5 (1.6 , 17.4)*	8.5 (2.8 , 14.1)**	3.0 (−3.8 , 9.8)	−7.2 (−22.4 , 8.1)
Maximum					1.1 (−9.5 , 11.7)
Late aggregation					
D0–6	3.7 (−5.4 , 12.9)	10.5 (0.8 , 20.3)*	8.1 (−1.2 , 17.3)	3.3 (−5.3 , 11.8)	−15.0 (−30.4 , 0.5)
D0–12	6.6 (−2.0 , 15.1)	11.6 (1.2 , 21.9)*	8.5 (−0.7 , 17.6)	7.5 (−2.3 , 17.2)	−14.1 (−29.0 , 0.8)
D0–24	1.7 (−6.8 , 10.2)	11.2 (1.4 , 21.0)*	8.9 (1.12 , 16.6)*	9.9 (−2.5 , 22.3)	−17.3 (−35.2 , 0.6)
I24–48	9.8 (2.4 , 17.2)*	7.5 (−2.2 , 17.1)	5.5 (−1.5 , 12.4)	1.9 (−9.0 , 12.7)	−18.4 (−39.0 , 2.2)
I48–72	6.4 (0.7 , 12.2)*	18.1 (8.4 , 27.8)**	7.9 (2.3 , 13.4)**	8.9 (2.6 , 15.2)**	−26.0 (−44.1 , −7.8)**
I72–96	8.2 (−1.2 , 17.6)	4.2 (−5.5 , 13.9)	3.4 (−5.7 , 12.6)	4.8 (−4.3 , 13.9)	1.2 (−14.8 , 17.3)
D+I0–96	18.1 (9.1 , 27.1)**	20.4 (8.4 , 32.4)**	13.0 (4.9 , 21.1)**	16.1 (5.0 , 27.2)**	−17.1 (−40.8 , 6.7)
Maximum					−16.4 (−31.0 , −1.8)*

Data are percent change of 5 µmol/ADP-induced maximal platelet aggregation and late aggregation (6 min after maximum), with 95% C.I. in parentheses. Values are based on hourly measurements from a monitor located within the city center of Rotterdam. Blood was drawn from all subjects 09^{00} and 11^{00} hours. *$p < 0.05$, **$p < 0.01$.

Thrombin generation

We observed a significant increase in ETP for the gaseous pollutants CO, NO, and NO2 at time lags representing indirect effects of air pollution (I24–48, +2%/IQR of NO and +4%/IQR of NO2; I72–96, +3%/IQR of CO and +2%/IQR of NO; all $p < 0.05$) and a significant increase in peak thrombin generation (I24–48, +4%/IQR of NO and +8%/IQR of NO2, both $p < 0.01$; I72–96, +4%/IQR of NO, $p < 0.05$) (Table 2.4). In addition, peak thrombin generation was significantly increased by 6% per IQR of maximal daily concentration of O3 (Table 2.4).

Table 2.4. *Estimated changes of thrombin generation associated with mean air pollutant levels at various time lags preceding blood samplings*

	Air pollutant				
Time lag	PM10	CO	NO	NO_2	O_3
ETP					
D0–6	−0.2 (−2.6 , 2.1)	−1.5 (−3.7 , 0.8)	−0.4 (−2.3 , 1.5)	−1.2 (−3.6 , 1.2)	3.2 (−0.3 , 6.7)
D0–12	−0.2 (−2.2 , 1.8)	−1.1 (−3.4 , 1.1)	−0.4 (−2.0 , 1.1)	−0.2 (−2.8 , 2.4)	0.8 (−2.8 , 4.4)
D0–24	−0.7 (−2.7 , 1.4)	−1.5 (−3.9 , 0.9)	−0.3 (−2.2 , 1.6)	0.3 (−2.5 , 3.1)	0.3 (−3.8 , 4.5)
I24–48	−0.2 (−2.3 , 1.9)	−0.7 (−3.4 , 2.0)	1.9 (0.1 , 3.7)*	3.5 (0.2 , 6.8)*	−0.9 (−5.4 , 3.6)
I48–72	1.9 (−0.2 , 4.0)	0.8 (−1.9 , 3.4)	0.8 (−0.8 , 2.5)	2.1 (−1.0 , 5.2)	−0.6 (−4.5 , 3.3)
I72–96	1.5 (−1.1 , 4.2)	3.5 (0.8 , 6.2)*	2.1 (0.2 , 4.0)*	0.8 (−1.9 , 3.5)	−0.6 (−4.1 , 3.0)
D+I0–96	0.7 (−2.3 , 3.8)	0.8 (−2.7 , 4.3)	1.7 (−1.1 , 4.5)	1.1 (−1.7 , 4.0)	1.0 (−3.2 , 5.2)
Maximum					2.3 (−1.2 , 5.8)
Peak Height					
D0–6	−0.8 (−4.8 , 3.1)	−2.5 (−6.3 , 1.3)	−0.4 (−3.6 , 2.8)	−1.5 (−5.5 , 2.5)	5.7 (−0.2 , 11.7)
D0–12	−0.7 (−4.1 , 2.6)	−1.9 (−5.7 , 1.9)	−0.4 (−3.1 , 2.3)	−0.7 (−5.1 , 3.7)	2.8 (−3.4 , 8.9)
D0–24	−1.6 (−5.0 , 1.8)	−3.3 (−7.3 , 0.7)	−0.6 (−3.9 , 2.6)	−0.6 (−5.3 , 4.1)	2.6 (−4.8 , 9.9)
I24–48	−0.2 (−3.7 , 3.3)	−1.3 (−6.1 , 3.6)	4.1 (1.1 , 7.2)**	8.0 (2.4 , 13.6)**	0.2 (−7.3 , 7.8)
I48–72	1.9 (−1.6 , 5.4)	−0.5 (−5.0 , 4.0)	1.2 (−1.6 , 4.0)	3.7 (−1.5 , 9.0)	1.4 (−5.1 , 8.0)
I72–96	0.2 (−4.2 , 4.6)	3.8 (−0.8 , 8.4)	3.5 (0.4 , 6.7)*	−0.2 (−4.8 , 4.4)	2.6 (−3.3 , 8.5)
D+I0–96	−1.9 (−6.9 , 3.2)	−1.7 (−7.5 , 4.2)	3.1 (−1.7 , 7.8)	1.0 (−4.1 , 6.0)	6.6 (−0.7 , 13.8)
Maximum					6.3 (0.3 , 12.3)*
Lag time					
D0–6	0.1 (−1.4 , 1.7)	1.0 (−0.5 , 2.5)	−0.1 (−1.4 , 1.1)	0.0 (−1.6 , 1.6)	−0.3 (−2.6 , 2.0)
D0–12	0.2 (−1.2 , 1.5)	1.0 (−0.5 , 2.5)	0.0 (−1.1 , 1.0)	0.0 (−1.8 , 1.7)	−0.4 (−2.7 , 2.0)
D0–24	1.1 (−0.2 , 2.4)	1.6 (0.1 , 3.1)*	0.6 (−0.6 , 1.8)	0.2 (−1.6 , 2.0)	−0.3 (−2.9 , 2.3)
I24–48	0.1 (−1.3 , 1.5)	0.4 (−1.3 , 2.2)	−1.8 (−2.9 , −0.7)**	−3.1 (−5.1 , −1.0)**	3.0 (0.4 , 5.7)*
I48–72	0.0 (−1.4 , 1.4)	−1.0 (−2.7 , 0.7)	−0.8 (−1.8 , 0.3)	−2.5 (−4.3 , −0.6)*	1.2 (−1.1 , 3.6)
I72–96	0.6 (−1.2 , 2.3)	−1.5 (−3.2 , 0.2)	−1.4 (−2.6 , −0.2)*	0.0 (−1.8 , 1.7)	0.6 (−1.6 , 2.8)
D+I0–96	1.2 (−0.7 , 3.2)	0.1 (−2.1 , 2.2)	−1.1 (−2.8 , 0.5)	−0.7 (−2.5 , 1.1)	0.6 (−2.0 , 3.2)
Maximum					−1.2 (−3.5 , 1.0)

Percent change of thrombin with their 95% C.I. in parentheses. Thrombin generation was induced with 1 pM tissue factor and 4 μmol/L phospholipids. Values are based on hourly measurements from a monitor located within the city center of Rotterdam. Blood was drawn from all subjects 09^{00} and 11^{00} hours. *$p < 0.05$, **$p < 0.01$.

The associations with PM10 levels were less clear and not statistically significant, although the estimates for time lags that represent indirect effects on ETP were mainly positive (Fig. 2. 4A). The lag time of thrombin generation was significantly lower when the concentrations of gaseous pollutants were increased at time lags representing indirect effects (I24–48, −2%/IQR of NO and −3%/IQR of NO2, both $p < 0.01$; I48–72, −2%/IQR of NO2, $p < 0.05$; I72–96, −1%/IQR of NO, $p < 0.05$), except for the time lag D0–24 (+2%/IQR of CO, $p < 0.05$) (Table 2.4). We observed no clear associations between PM10 and peak height or lag time of thrombin generation (Fig. 2. 4B,C). The associations between air pollutants and parameters of thrombin generation induced by 5 pM TF showed similar associations (data not shown). Similar results for thrombin generation parameters were also obtained when we performed the analyses in nonsmokers only or after excluding women using oral contraceptives (data not shown).

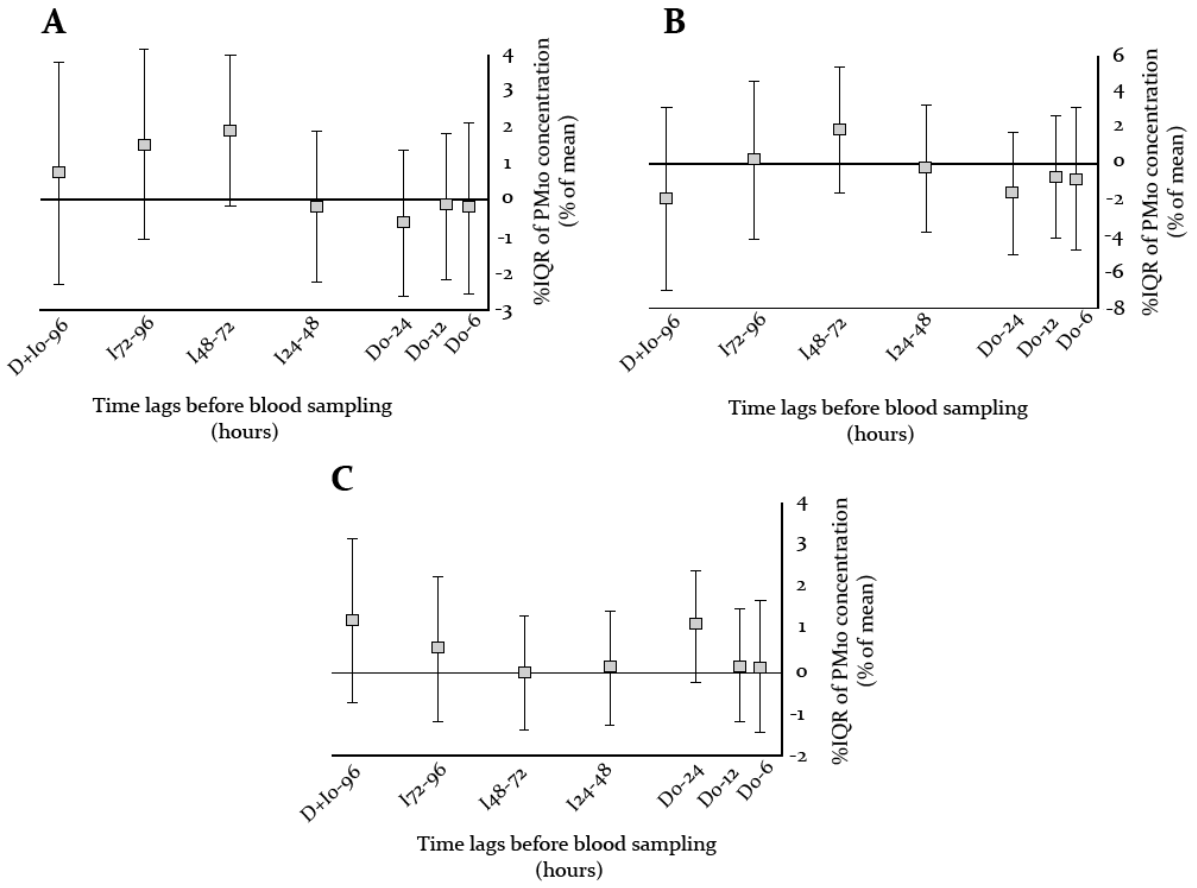


Figure 2.4. ***Effects of PM10 on thrombin generation***
Estimated effects of PM10 on endogenous thrombin potential (*A*), peak height (*B*) and lag time (C) are given as percent change from the mean of each individual per increase in interquartile of PM10 concentration. Direct effects of PM10 on thrombin generation are represented by time lags of D0–6, D0–12, and D0–24, whereas indirect effects are represented by time lags I24–48, I48–72, and I72–96. The effect of 4 day mean concentration of PM10 on thrombin generation is represented by time lag D+I0–96.

Inflammation

Our data suggest that there are no indirect effects of PM10 on inflammation, because we found no statistically significant associations between PM10 concentrations and either CRP or fibrinogen levels (Fig. 2. 5). We obtained similar results for CO, NO, NO2, and O3 (Table 2.5) and when we performed the analyses in nonsmokers only or after excluding women using oral contraceptives (data not shown).

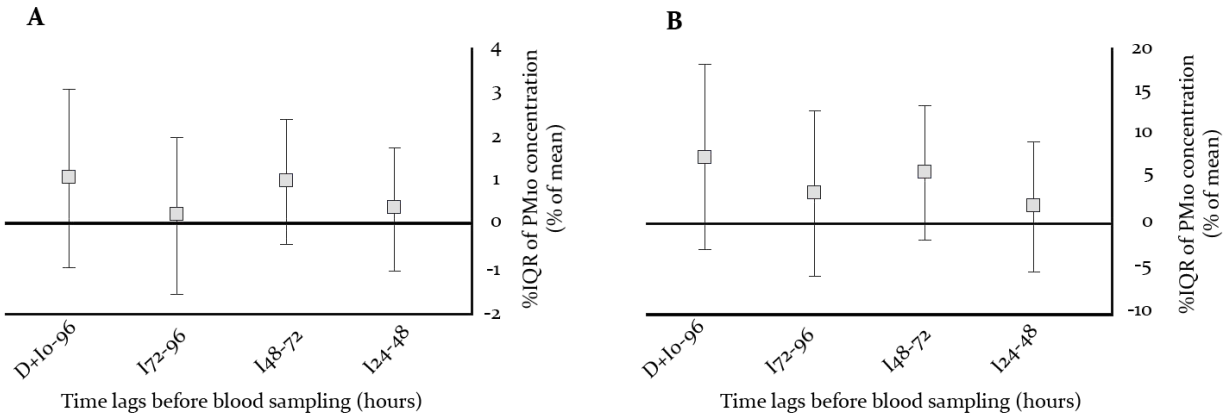


Figure 2.5. ***Indirect effects of PM10 on inflammation***
Estimated indirect effects of PM10 on fibrinogen (A) and C-reactive protein (B) are given as percent change from the mean of each individual per increase in interquartile of PM10 concentration. These indirect effects of PM10 on inflammation are represented by time lags I24–48, I48–72, and I72–96. The effect of 4 day mean of PM10 on inflammation is represented by time lag D+I0–96.

Table 2.5. *Estimated changes of inflammatory markers associated with mean air pollutant levels time lags representing indirect effects preceding blood samplings*

	Air pollutant				
Time lag	PM10	CO	NO	NO_2	O_3
Fibrinogen					
I24–48	0.4 (–1.1, 1.8)	0.0 (–1.7, 1.8)	0.1 (–1.0, 1.3)	0.4 (–1.7, 2.5)	–0.6 (–3.2, 2.1)
I48–72	1.0 (–0.5, 2.4)	0.0 (–1.8, 1.9)	0.3 (–0.8, 1.4)	1.4 (–0.6, 3.4)	–1.4 (–3.8, 1.0)
I72–96	0.2 (–1.6, 2.0)	–0.1 (–1.9, 1.7)	0.1 (–1.1, 1.4)	–0.4 (–2.3, 1.4)	0.5 (–1.7, 2.8)
C-Reactive Protein					
I24–48	1.9 (–5.6, 9.4)	3.2 (–6.4, 12.8)	3.6 (–2.9, 10.0)	6.5 (–4.9, 17.8)	–0.5 (–14.7, 13.8)
I48–72	5.8 (–2.0, 13.5)	–1.9 (–12.5, 8.7)	0.1 (–6.5, 6.7)	–0.1 (–11.0, 10.8)	3.7 (–9.7, 17.2)
I72–96	3.4(–6.2, 12.9)	–4.5 (–15.3, 6.3)	-4.6 (–12.0, 2.9)	–6.9 (–17.2, 3.5)	5.9 (–6.8, 18.7)

Percent change with 95% C.I. in parentheses. Values are based on hourly measurements from a monitor located within the city center of Rotterdam.

Discussion

The main observation of this study was that air pollution (except O3) was associated with increased platelet aggregation and increased thrombin generation, but not with the inflammatory markers fibrinogen and CRP. The significant associations between various air pollutants and increased maximal aggregation and late aggregation for time lags within 24–96 h before blood sampling suggest that exposure to air pollution indirectly increases blood thrombogenicity. These indirect effects may be the result of air pollution–induced synthesis of TF [22], which can increase *in vivo* platelet reactivity [23]. TF bearing microparticles have also been suggested to contribute to these effects on platelets either directly or indirectly via increased blood coagulability [24]. Another possible mechanism of indirect platelet activation might reside in pulmonary oxidative stress and the activation of subsets of white blood cells that lead to a systemic lowering of endothelial and platelet derived nitrogen oxide and concomitant platelet activation [25]. Our results also indicate that it is unlikely that blood platelets are directly activated by contact with artificial surfaces of PM because we observed no association between platelet aggregation and PM concentrations for time lags representing direct effects. We confirmed these findings with our *in vitro* experiments, in which we saw no direct effects (0–2 h) of the addition of PM on platelet aggregation.

Results for thrombin generation suggest that air pollution leads to an overall tendency toward a hypercoagulable state, because both ETP and peak thrombin generation were increased after exposure to higher levels of gaseous air pollutants. Again, these indirect effects on thrombin generation may be caused by elevated levels of TF. Notably, only gaseous pollutants, and not PM10, were associated with these indirect effects. The gaseous air pollutants, especially NO2 and CO, can be considered markers for motor vehicle traffic and have been shown to be highly correlated with ultrafine particles [26]. It is mainly this subset of ultrafine particles from the overall PM air pollution that has an effect on thrombin generation [27]. This could explain why only the gaseous pollutants, and not PM10 mass concentration, was associated with thrombin generation in this study. Another mechanism may involve altered synthesis of pro- and anticoagulant proteins. However, previous studies on air pollution–related changes in plasma levels of PT, factor VII, antithrombin, protein C, and protein S are conflicting [5, 28-30]. In our study, changes in thrombin generation were independent of the changes in fibrinogen concentrations.

Because thrombin generation depends not only on fibrinogen but also on other pro- and anticoagulant proteins, this suggests that air pollution can induce changes in hemostatic balance that are not inflammation driven. In our study, air pollution was not associated with systemic inflammation. We measured fibrinogen and CRP, two sensitive inflammatory markers that are consistently associated with cardiovascular risk. The results from epidemiologic studies on air pollution and inflammation are conflicting, as are those from laboratory studies on the inflammatory responses in human volunteers or animals after experimentally controlled exposure to air pollution [28, 30-33]. Because fibrinogen and CRP do not reflect all aspects of inflammation, measuring other inflammatory markers, such as interleukin 6 and tumor necrosis factor-α, may still reveal associations between air pollution and inflammation [5, 34].

Most of the significant associations observed in the present study concern more than one air pollutant, especially in the case of late aggregation, which was associated with all studied air pollutants. The correlation coefficients between the concentrations of air pollutants were moderate and mainly positive (> 0.6), except between O_3 and the other air pollutants (0.4 to 0.6). This probably explains the observed opposite effects of O_3 on most of the plasma variables, compared with the effects of PM10, CO, NO, and NO_2. However, when we analyzed these effects of O_3 in a two pollutant model with PM10, only the effects of PM10 remained statistically significant with similar estimates (data not shown), suggesting that PM10, rather than O_3, is responsible for the observed effects on platelet aggregation and thrombin generation. This was in contrast to the results of the two pollutant models that combined PM10 with CO, NO, or NO_2, which indicated that effects of these gases are mainly independent of the effects of PM10 (data not shown). Nevertheless, it remains difficult to completely discern causal air pollutant(s) from their surrogate markers.

The present study was designed to represent a real life urban situation. Our approach combines several strengths. First, we have evaluated the effects of air pollution on blood parameters over a period of 1 year. In this longitudinal study design, participants were their own controls, which ensure the most reliable estimates of acute effects of exposure to air pollution. Second, we selected subjects with a similar exposure to local air pollution. Third, several studies have shown that central site measurements correlate well with personal exposures for longitudinal acute effects [35-37]. Although the study participants have spent a significant amount of time living or working in Rotterdam during the study period, their estimated exposure to air pollution may vary. However, we did not design our study to correct for these possible variations. Another limitation of the study is that the PM10 mass

concentration is a poor measure of its biological activity, because the corresponding particles are heterogeneous in composition (e.g., endotoxins and metals) and may therefore trigger different biological responses [3]. All subjects in this study were healthy volunteers.The observed effects of air pollution on hemostasis may differ (being possibly more pronounced) in subjects with coronary artery disease or subjects at higher risk for this disease. In conclusion, our data show a significant association between exposure to air pollution and systemic prothrombotic tendency of the blood via increased platelet aggregation and thrombin generation in a healthy population. This association may point to a relevant biological mechanism that contributes to the risk association between air pollution and cardiovascular disease.

References

1 McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, Harrington R, Svartengren M, Han IK, Ohman-Strickland P, Chung KF, Zhang J. Respiratory effects of exposure to diesel traffic in persons with asthma. *N Engl J Med*. 2007; **357**: 2348-58.

2 Peng RD, Chang HH, Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Coarse particulate matter air pollution and hospital admissions for cardiovascular and respiratory diseases among Medicare patients. *Jama*. 2008; **299**: 2172-9.

3 Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*. 2004; **109**: 2655-71.

4 Peters A, Doring A, Wichmann HE, Koenig W. Increased plasma viscosity during an air pollution episode: a link to mortality? *Lancet*. 1997; **349**: 1582-7.

5 Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med*. 2006; **173**: 432-41.

6 Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation*. 2002; **105**: 411-4.

7 Nemmar A, Hoylaerts MF, Hoet PH, Nemery B. Possible mechanisms of the cardiovascular effects of inhaled particles: systemic translocation and prothrombotic effects. *Toxicol Lett*. 2004; **149**: 243-53.

8 Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol*. 2005; **146**: 882-93.

9 Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet*. 1995; **345**: 176-8.

10 Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ Health Perspect*. 2001; **109 Suppl 4**: 523-7.

11 Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med*. 1999; **159**: 702-9.

12 Brook RD. Is air pollution a cause of cardiovascular disease? Updated review and controversies. *Rev Environ Health*. 2007; **22**: 115-37.

13 Gong H, Jr. Health effects of air pollution. A review of clinical studies. *Clin Chest Med*. 1992; **13**: 201-14.

14 National Institute for Public Health and the Environment (RIVM). 2009. Milieuportaal voor professionals. Available: www.lml.rivm.nl/data/smog/index.html)[accessed 5 August 2011].

15 van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Reneman RS, oude Egbrink MG. In vivo blockade of platelet ADP receptor P2Y12 reduces embolus and thrombus formation but not thrombus stability. *Arterioscler Thromb Vasc Biol*. 2003; **23**: 518-23.

16 Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. *J Clin Invest*. 2001; **107**: 1503-5.

17 Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Beguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003; **33**: 4-15.

18 Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost*. 2008; **6**: 125-31.

19 ten Cate-Hoek AJ, Dielis AW, Spronk HM, van Oerle R, Hamulyak K, Prins MH, ten Cate H. Thrombin generation in patients after acute deep-vein thrombosis. *Thromb Haemost*. 2008; **100**: 240-5.

20 Wood SN. GAMs and generalized ridge regression for R. *R News* 2001; **1**: 20-5.

21 Ruckerl R, Greven S, Ljungman P, Aalto P, Antoniades C, Bellander T, Berglind N, Chrysohoou C, Forastiere F, Jacquemin B, von Klot S, Koenig W, Kuchenhoff H, Lanki T, Pekkanen J, Perucci CA, Schneider A, Sunyer J, Peters A. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environ Health Perspect*. 2007; **115**: 1072-80.

22 Sun Q, Yue P, Kirk RI, Wang A, Moatti D, Jin X, Lu B, Schecter AD, Lippmann M, Gordon T, Chen LC, Rajagopalan S. Ambient air particulate matter exposure and tissue factor expression in atherosclerosis. *Inhal Toxicol*. 2008; **20**: 127-37.

23 Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor-and collagen-dependent thrombus formation in arterioles and venules in vivo differential sensitivity to thrombin inhibition. *Microcirculation*. 2008; **15**: 269-82.

24 Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol*. 2006; **26**: 2594-604.

25 Brook RD. Air pollution: what is bad for the arteries might be bad for the veins. *Arch Intern Med*. 2008; **168**: 909-11.

26 Cyrys J, Stolzel M, Heinrich J, Kreyling WG, Menzel N, Wittmaack K, Tuch T, Wichmann HE. Elemental composition and sources of fine and ultrafine ambient particles in Erfurt, Germany. *Sci Total Environ*. 2003; **305**: 143-56.

27 Kilinç E, van Oerle R, Borissoff JI, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HM. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *J Thromb Haemost*. 2011; **9**: 1359-67.

28 Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost*. 2007; **5**: 252-60.

29 Pekkanen J, Brunner EJ, Anderson HR, Tiittanen P, Atkinson RW. Daily concentrations of air pollution and plasma fibrinogen in London. *Occup Environ Med*. 2000; **57**: 818-22.

30 Seaton A, Soutar A, Crawford V, Elton R, McNerlan S, Cherrie J, Watt M, Agius R, Stout R. Particulate air pollution and the blood. *Thorax*. 1999; **54**: 1027-32.

31 Chuang KJ, Chan CC, Su TC, Lee CT, Tang CS. The effect of urban air pollution on inflammation, oxidative stress, coagulation, and autonomic dysfunction in young adults. *Am J Respir Crit Care Med*. 2007; **176**: 370-6.

32 Maier KL, Alessandrini F, Beck-Speier I, Hofer TP, Diabate S, Bitterle E, Stoger T, Jakob T, Behrendt H, Horsch M, Beckers J, Ziesenis A, Hultner L, Frankenberger M, Krauss-Etschmann S, Schulz H. Health effects of ambient particulate matter--biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhal Toxicol*. 2008; **20**: 319-37.

33 Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. *Am J Respir Crit Care Med*. 2003; **168**: 1366-72.

34 Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest*. 2007; **117**: 2952-61.

35 Janssen NA, Hoek G, Brunekreef B, Harssema H, Mensink I, Zuidhof A. Personal sampling of particles in adults: relation among personal, indoor, and outdoor air concentrations. *Am J Epidemiol*. 1998; **147**: 537-47.

36 Janssen NA, Lanki T, Hoek G, Vallius M, de Hartog JJ, Van Grieken R, Pekkanen J, Brunekreef B. Associations between ambient, personal, and indoor exposure to fine particulate matter constituents in Dutch and Finnish panels of cardiovascular patients. *Occup Environ Med*. 2005; **62**: 868-77.

37 World Health Organization. Particulate matter, ozone, nitrogen dioxide and sulfur dioxide. In: *Air Quality Guidelines: Global Update 2005.* Copenhagen:World Health Organization, Regional Office for Europe, 76–100., 2006.

CHAPTER 3

PULMONARY AND CARDIOVASCULAR EFFECTS OF TRAFFIC RELATED PARTICULATE MATTER: 4 WEEK EXPOSURE OF RATS TO ROADSIDE AND DIESEL ENGINE EXHAUST PARTICLES

Based on:

Gerlofs-Nijland, M.E., Totlandsdal, A.I., Kilinç, E., Boere, A.J.F., Fokkens, P.H.B., Leseman, D.L.A.C., Sioutas, C., Schwarze, P.E., Spronk, H.M., Hadoke, P.W.F., Miller, M.R., Cassee, F.R.

2010. Pulmonary and cardiovascular effects of traffic-related particulate matter: 4-week exposure of rats to roadside and diesel engine exhaust particles. *Inhalation Toxicology*. 22(14):1162-1173.

Abstract

Traffic-related particulate matter (PM) may play an important role in the development of adverse health effects, as documented extensively in acute toxicity studies. However, rather little is known about the impacts of prolonged exposure to PM.
We hypothesized that long-term exposure to PM from traffic adversely affects the pulmonary and cardiovascular system through exacerbation of an inflammatory response.

To examine this hypothesis, Fisher F344 rats, with a mild pulmonary inflammation at the onset of exposure, were exposed for 4 weeks, 5 days/week for 6 hours a day to: a) diluted diesel engine exhaust (PM_{DEE}), or: b) near roadside PM ($PM_{2.5}$). Ultrafine particulates, which are largely present in diesel soot, may enter the systemic circulation and directly or indirectly trigger cardiovascular effects. Hence, we assessed the effects of traffic-related PM on pulmonary inflammation and activity of procoagulants, vascular function in arteries, and cytokine levels in the heart 24 hours after termination of the exposures.

No major adverse health effects of prolonged exposure to traffic-related PM were detected. However, some systemic effects due to PM_{DEE} exposure occurred including decreased numbers of white blood cells and reduced von Willebrand factor protein in the circulation. In addition, lung tissue factor activity is reduced in conjunction with reduced lung tissue thrombin generation.

To what extent these alterations contribute to thrombotic effects and vascular diseases remains to be established. In conclusion, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms.

Introduction

Airborne ambient particulate matter (PM) is considered to play an important role in the adverse health effects associated with air pollution [1]. Most epidemiological studies have focused on the effects of short-term exposure to air pollutants. In these short-term studies a clear link was shown between levels of air pollutants and a tendency towards a hypercoagulable state. These associations are, for example, found with both PM_{10} and NO_2 [2] or only with the traffic related gaseous (NO_2 and CO) instead of with PM mass [3]. However, several epidemiological studies have associated long-term exposure to the fine fraction of PM ($PM_{2.5}$: particulate matter with an aerodynamic diameter below 2.5 μm) with an increase in pulmonary and cardiovascular morbidity and mortality [4, 5]. Notably, living close to a busy road over several years has been associated with increased cardiopulmonary mortality [6, 7]. Hence, road traffic, which is a major source of $PM_{2.5}$ in urban areas, could be particularly responsible for the impact of PM exposure on human health [5, 8-10].

These epidemiological observations are supported by controlled toxicology studies performed with animals and human volunteers exposed to PM samples from different sites. Animal exposure studies attribute a greater toxicity of PM collected at locations that contain a high proportion of traffic emissions [11-13]. Interestingly, it is becoming evident that exposure to traffic-related PM has marked actions on the cardiovascular system, as well as their more well-recognized pulmonary effects [14, 15]. Short-term exposures to diesel engine exhaust, an important source of $PM_{2.5}$, cause both vascular dysfunction and impaired endogenous fibrinolysis in healthy and compromised volunteers [16, 17]. In addition, elevated thrombus formation was shown ex vivo after inhalation of diesel engine exhaust [18].

In these studies, mostly fresh generated emission particles were used as a surrogate of PM, which are not necessarily representative of the PM in ambient air. Inhalation of elevated concentrations of ambient air particles collected with different size ranges at different sites by using concentrator technology [19-21] represents a more realistic PM exposure [22]. A few hours exposure to $PM_{2.5}$ from urban traffic sites caused an increase in cardiovascular symptoms and in lung toxicity and inflammation in rodents and volunteers [23-29]. This also suggests a major contribution of traffic-related particles to the biological effects associated with PM. Since these studies, in which relatively high PM exposure levels were applied, will be useful in understanding the impact of episodic PM exposure on human health. At present, only a few publications, all from the same well-conducted study in New York, describe the impact of prolonged exposure on normal and susceptible (i.e. mimicking a human disease)

rodents at lower concentration, more environmentally-relevant levels [30-36]. The most striking results were seen on the cardiovascular system with altered vasomotor tone, induced vascular inflammation, and potentiated atherosclerosis both in Sterling Forrest [34] and more traffic-influenced Manhattan [29].

In order to investigate the contribution of traffic to the long-term effect of particles, we performed a series of experiments in which we exposed rats to filtered air, to diluted diesel engine exhaust (rich in ultrafine particles) and to $PM_{2.5}$ derived from a nearby very busy freeway. Prior to the PM exposures, a minor lung inflammation was induced by exposure exposing the rats to ozone. We hypothesized that prolonged (i.e. 4-week) exposure to traffic-derived $PM_{2.5}$ exacerbates the existing inflammatory reaction, which could result in an induction of oxidative stress with subsequent effects on the pulmonary and cardiovascular system. In order to verify this hypothesis, a comprehensive analysis of markers for pulmonary (oxidative stress, cytotoxicity, inflammation) and cardiovascular (coagulation, fibrinolysis, endothelial damage, thrombogenicity, heart inflammation, aorta contractibility) effects due to exposure to traffic-derived PM was performed.

Matherials and Methods

Animals

Male SPF F344 (DUCRL) rats were obtained from Charles River (Germany). The rats were housed in macrolon type III cages with a room temperature maintained at 22 ± 2 °C, relative humidity at 40-70%, and a 12-h light/dark cycle. Rats were allowed access to a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, the Netherlands) and tap water via drinking bottles ad libitum during non-exposure periods. Exposure started after 7 days of acclimatization.

Experimental Design

A total of three experiments were conducted using different types of PM exposure [37]. At day 0, all rats were exposed (whole body) for 12 h to 0.4 ppm ozone [38] to initiate a minor inflammation in the lung [24]. After the initial ozone exposure the animals were transferred to RIVM's mobile exposure laboratory (MAPCEL) and subsequently exposed for 4 weeks (5 days per week, 6 h per day) to diesel engine exhaust (PM_{DEE}) or to concentrated ambient particles with an aerodynamic diameter <2.5 μm ($PM_{2.5}$ or also known as CAPs) near a busy roadside at Utrecht, the Netherlands (Fig. 3.1). The $PM_{2.5}$ roadside study was repeated once due to the inherent variability of the mass and composition of ambient PM.

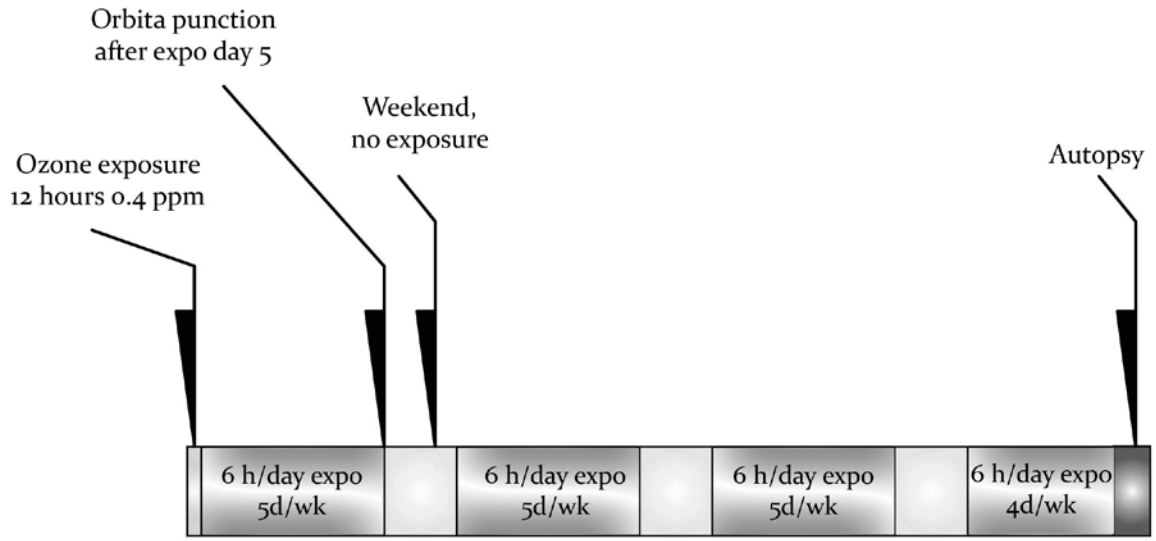


Figure 3.1. ***Experimental exposure design***

PM_{DEE}. Exposure was performed using a 35 KVA diesel generator (Bredenoord, Apeldoorn, the Netherlands) under idling conditions. The animals (n=15/group) were exposed to 150 μg/m^3 PM_{DEE} diluted with clean conditioned air.

$PM_{2.5}$ *roadside*. Rats were exposed to increased levels of $PM_{2.5}$ using the Versatile Aerosol Concentration Enrichment Systems (VACES) [19, 20] with a theoretical enrichment factor of 20 and at an output flow rate directed into the nose-only system of 20 LPM. The MAPCEL was placed close to (15 meter), and east of a major roadside (A2, Utrecht-Amsterdam), with prevailing westerly winds, used by 160,000 cars and trucks per day.

Control animals were exposed to filtered, purified air with the same temperature and relative humidity as the test atmospheres. All rats were nose-only exposed using novoplast tubes T (Münster AG, Muttenz, Switzerland) in nose-only exposure chambers. One week before exposure, animals were trained in nose-only tubes to reduce the stress of the restraint (3 days, 1h per day). Immediately after the exposures, the animals were returned to their housing facilities. Blood (1 ml) was obtained after the first week of exposure (directly after the fifth exposure day; Figure 1) by orbital puncture under Brevimethal anaesthesia (50 mg/kg bodyweight, i.m.) to measure fibrinogen, von Willebrand factor, PAI-1 and CC16. Based on the initial findings, additional parameters were investigated, i.e. vascular function, measurement of cytokines in cardiac tissue, and tissue factor activity and thrombin generation in lung tissue

in one of the two roadside experiments to gain more insight in a possible biological mechanism.

Necropsy was performed on the day after the last exposure day (Figure 1). Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI, Bilthoven, Netherlands).

Characterization of the test atmospheres

A condensation particle counter (CPC model 3022A, TSI St. Paul, Minn., USA) was used to determine the particle number concentration in the inlet of the exposure chamber. The mass concentration was measured continuously in the inlet of the exposure chamber during the exposure with a nephelometer (DATARAM 2000, MIE, Billerica, Mass., USA). In the $PM_{2.5}$ roadside experiments, the particle number and mass concentration were measured both before the VACES inlet and after the VACES. The time-integrated PM concentrations were also measured in the inlet of the exposure chamber by means of collection on three 47-mm filters placed in parallel, two PolyTetraFluoroEthylene (Teflo R2PJ047, Pall Corp. Ann Arbor Mi, USA) and one Quartz filter (QMA, Whatman Int Inc Maidstone England). A carbon sampler tube (Anasorb CSC Lot 2000, SKC Inc., Eighty Four, PA USA) was placed downstream of one of the PTFE filters at the outlet to collect the volatile organic components (VOC). One set of PTFE filters and a carbon sampler tube were used for each exposure week. Carbon monoxide (ML 9830 CO, Lear Siegler, Englewood Co, USA), sulfur dioxide (Model 43A, Thermo Environmental Instruments, Franklin MA, USA) and nitrogen oxides (Model 42W, Thermo Environmental Instruments, Franklin MA, USA) were measured in the PM_{DEE} mixing chamber or at the inlet of the VACES. In the PM_{DEE} experiment, a Scanning Mobility Particle Sizer (SMPS, DMA model 3071 + CPC model 3022A, TSI St. Paul, Minn., USA) was used to measure the particle size distribution (mean diameter and geometric standard deviation) every hour in the inlet of the exposure chamber. The weekly time-integrated particle size mass distribution was measured at the inlet of the VACES with an eight-stage Micro Orifice Impactor (Model No. 100, MSP Corporation, Minneapolis Mn, USA). Temperature and relative humidity were recorded once every 5 minutes in the exposure chamber and control exposure chamber and recorded every 30 minutes in the inlet of the exposure chamber. The activated carbon samplers were analyzed using GC-MS (RIVM, Bilthoven, the Netherlands) to determine the VOC concentrations.

Necropsy

The day after the final test atmosphere exposure, the rats were anesthetized with a mix of Ketamine and Rompun; 100 mg /kg of Ketamine (Aesculaap, Boxtel, the Netherlands) and 1 mg/kg Rompun (Bayer, Leverkusen, Germany). A cannula was inserted in the trachea. The abdomen was opened and a minimum of 6 ml blood was sampled through the abdominal aorta. The chest was opened and the lungs were perfused (pressure 30 cm H_2O) with saline to remove the blood from the lung using a cannula placed through the right heart chamber into the pulmonary artery. The left bronchus was clamped and the left lung was cut just behind the clamp. The left lung was weighed and fixed for 1 h under a constant pressure of 20 cm H_2O using 4% phosphate-buffered formaldehyde. The right lung was used for bronchoalveolar lavage fluid (BALF) collection by 3 lavages of sterline saline (27 ml/kg body weight). The heart was dissected, split into the right and left side and frozen in liquid N_2. The descending thoracic aorta was dissected and immediately placed in Krebs buffer for organ bath measurements.

Bronchoalveolar lavage analyses

The collected BALF was centrifuged at 400 × g, 4°C, for 10 min. The cell-free fluid from the lavage was used for assessment of lactate dehydrogenase (LDH, marker for cytotoxicity), *N*-acetylglucosaminidase (NAG, macrophage activation), alkaline phosphatase (ALP, type II cell damage), and the levels of Clara-cell 16 protein (CC16, lung cell damages), reduced glutathione and oxidized glutathione (GSH and GSSG respectively), albumin and total protein levels (increased permeability of the alveolar–capillary barrier), inflammatory mediators interleukin 6 (IL-6), and tumor necrosis factor (TNF)-α were determined as previously described [24, 39]. Heme-oxygenase (HO)-1, a marker of oxidative stress, was determined using a commercially obtained reagent kit (Roche Nederland B.V, Mijdrecht, the Netherlands). The BALF pellet was resuspended in saline and used for total cell counts as well as preparation of cytospins for cell differential counts as previously described [39].

Hematological Analyses

Plasma levels of fibrinogen and CC16 were determined as previously described [24, 40]. Von Willebrand Factor (vWF) was measured by enzyme-linked immunosorbent assay (ELISA; American Diagnostica Inc., Stamford, US). Levels of tissue plasminogen activator (tPA) total antigen and active plasminogen activator inhibitor (PAI)-1 were measured in citrated plasma by ELISA (Innovative Research, Michigan, USA). Cell differentials were determined in EDTA

(K3) (Terumo Europe N.V., Leuven, Belgium) anticoagulated blood in a H1-E multispecies hematology analyzer (Bayer B.V., Mijdrecht, the Netherlands). The following parameters were measured: white and red blood cell concentrations (WBC and RBC, respectively), hemoglobin (HGB) and platelet (PLT) concentrations, the mean platelet volume (MPV), and the hematocrit value (HCT). In addition, mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet component (MPC) and hemoglobin distribution width (HDW) were provided.

Pathology

The left lung was embedded in paraffin after fixation with formaldehyde. Tissues were cut in 5 μm slices and slides were stained with hematoxylin and eosin before light microscopic examination. Slides were screened for pathological changes as a result of the exposure. The pathological lesions and inflammation were semi-quantitatively and blindly scored as absent, minimal, slight, moderate, marked or strong.

Vascular function

Ex vivo endothelial function and vascular responses were measured in isolated thoracic aortic rings by a modified method of [41, 42]. Segments of thoracic aorta (~5 mm length) were cleaned of connective tissue and mounted in organ baths in Krebs buffer bubbled with 5% CO_2/95% O_2 at 37°C. A baseline tension of 14.7 mN was gradually applied over 10 min and vessels were allowed to equilibrate for a further 30 min. Vessel viability was confirmed by a contractile response on addition of 80 mM KCl, repeated 3 times. Concentration-response curves to phenylephrine (PE; 1 nM – 10 μM) were obtained and a concentration that produced 80% maximum contraction (EC_{80}; 0.1-1 μM) was chosen for each individual rat aortic ring. Following contraction, cumulative concentration-response curves were obtained for acetylcholine (ACh; endothelium-dependent vasodilator; 1 nM – 10 μM), sodium nitroprusside (SNP; endothelium-independent nitric oxide donor; 0.1 nM – 1 μM) and isoprenaline or verapamil (endothelium- and nitric oxide-independent vasodilators; 1 nM – 10 μM). At least 30 min washout was allowed before application of subsequent drugs.

Analyses of cardiac tissue

Cytokine mRNA expression

The frozen right heart halves were homogenised in lysis-buffer and total RNA isolated using a 'Absolutely RNA™ RT-PCR Miniprep kit' (Stratagene, La Jolla, CA, USA). mRNA in each sample was reverse-transcribed into cDNA on a PCR system 2400 (Perkin Elmer) by using a High Capacity cDNA Archive Kit from Applied Biosystems. Quantitative real-time (QRT) PCR was performed on triplicate samples, with 18S rRNA as an internal control, using the Applied Biosystems 7500 Real-Time PCR System, with pre-designed TaqMan Gene Expression Assays (IL-6, Rn00561420_m1; IL-1β, Rn00580432_m1; TNF-α, Rn 00562055_m1, 18S, Hs99999901_s1) and TaqMan Universal PCR Master Mix. The expression of each gene within each sample was normalised against 18S rRNA and expressed relative to a heart tissue sample from one of the control rats using the formula $2^{-(\Delta\Delta Ct)}$, in which ΔΔCt = (Ct mRNA – Ct 18S rRNA)sample – (Ct mRNA – Ct, 18S rRNA) sample control rat.

Phosphorylation of mitogen-activated protein kinases (MAPKs)

Right heart halves were homogenized in lysis-buffer (20 mM Tris-HCL pH+7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 µM leupetin, 1.5 µM aprotinin, 15 µM pepstatin A and 1% Triton-X) and examined by Western analysis. Protein concentration in the samples was determined by using the BioRad DC Protein Assay (BioRad Life Science, CA, USA). Proteins (12.5-25 µg/well) from the homogenized heart tissue samples were separated by 10 % SDS-PAGE and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for the respective phosphorylated kinases (p-ERK1/2, p-JNK1/2, p-p38) prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed using the Super-Signal® West Dura chemoluminiscence system (Pierce, Perbio Science, Sweden) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with Mild antibody stripping solution® from Chemicon International (Termecula, CA, USA), and re-probed with antibodies against total MAPK proteins (ERK1/2, JNK, p38). Optical quantification of the protein bands were performed by using the KODAK 1D Image Analysis Software.

Tissue factor activity and thrombin generation in lung tissue

Tissue factor (TF) activity and tissue specific thrombin generation by means of the Calibrated Automated Thrombogram (CAT, Thrombinoscope BV, Maastricht, The Netherlands) were determined in lung tissue homogenates as described previously [43]. Briefly, thrombin generation was measured in the presence of a final concentration of 5 pM TF and 4 μM phospholipids (PL, at 20:20:60 mol% PS:PE:PC) after addition of lung homogenates in human plasma and alternatively measurements were also implemented in the absence of both TF and PL. All TG results were normalized and expressed as percentage of normal pooled platelet poor plasma which was prepared from at least 80 healthy volunteers [44].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or standard error of mean (SEM). Vascular responses are expressed as percentage of the maximal contraction to phenylephrine (PE), where positive values represent vasodilatation and 100% vasodilatation represents a complete abolition of PE-induced tone. The outcomes of the BALF, blood analyses, TF activity and thrombin generation were compared using an unpaired Student's *t*-test. Statistical comparisons of vasodilator curves were carried out using two-way Analysis of Variance (ANOVA), or unpaired Student's *t*-test for comparisons of EC_{50} and maximum responses (estimated following linear regression of individual curves using Graphpad Prism V4.0b). $P<0.05$ was accepted as statistically significant.

Results

Ozone exposure

A separate group of ten animals was used to confirm that ozone exposure induced a minor lung inflammation. At 24 h after the ozone exposure, there was a significant increase in lung permeability, as shown by elevated protein (487 ± 141 compared to control levels of 159 ± 49 mg/L; $p<0.001$) and albumin (248 ± 99 versus 50 ± 14 mg/L in control group; $p<0.001$) levels in BALF. Ozone exposure also increased the percentage of polymorphonuclear neutrophils (PMN) in the alveolar region by approximately 2.5% (3.05 ± 2.55% versus 0.65 ± 0.95% for control), although this increase did not reach statistical significance ($p>0.05$).

Exposures characteristics

Diesel engine exhaust - The PM_{DEE} exposures were performed at an overall average particle mass of 174 ± 15 μg/m^3 (Table 3.1). The average particle size (geometric median diameter) was

76 nm with a geometric standard deviation of 1.95 nm as measured by SMPS, with an average particle number concentration of 434 000/cm^3. During the first exposure week the carbon sampler was used only for one day and the amount of VOC measured was 564 μg/m^3. In addition, the concentrations of gaseous pollutants CO, NO, NO_2 and NOx were measured, with mean concentrations of 3050, 1671, 918 and 2589 μg/m^3, respectively. Levels of VOC during the last three exposure weeks could not be measured due to an overload in the carbon sampler tubes.

$PM_{2.5}$ rooadside #1 – During the first $PM_{2.5}$ roadside study the overall average particle mass was 485 ± 150 μg/m^3 (Table 3.1). The average particle number concentration was 312 000/cm^3 with a mean aerodynamic particle size of 1.04 μm and geometric standard deviation of 0.31 (measured by MOI before the VACES). The mean VOC content measured was 254 μg/m^3, which was mainly driven by high levels of VOC (820 μg/m^3) during the first week of exposure. These appeared to be caused by high amounts of heptane, most probably due to a 2-stroke engine used for lawn mowing activities nearby. The concentrations of gaseous pollutants NO, NO_2 and NOx were 56, 71 and 127 μg/m^3, respectively.

$PM_{2.5}$ roadside #2.- The overall average particle mass in the second $PM_{2.5}$ roadside study was 214 ± 17 μg/m^3. The average particle number concentration was 231 000/cm^3 with an associated aerodynamic mean particle size of 1.52 μm and geometric standard deviation of 0.23 (measured by MOI before the VACES). The VOC content was 19 μg/m^3 with NO, NO_2 and NOx concentrations of 81, 61 and 142 μg/m^3, respectively.

Table 3.1. *Particle exposure characteristics of diesel engine exhaust and concentrated ambient particles near a roadside*

Experiment	Week no	Mass	Number	CO	NO	NO_2	NOx	VOC	Inorganics	MMAD†
		μg/m³	# 10^5/cm³	μg/m³	μg/m³	μg/m³	μg/m³	μg/m³	μg/m³	μm
	1	160	4.83	3515	2058	1128	3186	564	nd	nd
	2	162	4.44	3131	1671	937	2608	897‡	nd	0.29
PM_{DEE}	3	191	4.18	2945	1571	841	2413	5416‡	nd	0.24
	4	182	3.89	2689	1397	765	2162	4068‡	nd	0.17
	Average	174	4.34	3050	1671	918	2589	564		0.23
	1	484	3.72	nd	72	84	156	820	nd	1.18
	2	284	3.51	nd	65	76	141	70	nd	0.99
$PM_{2.5}$	3	528	1.71	nd	22	42	65	45	nd	1.01
roadside #1	4	643	3.53	nd	69	82	151	80	nd	0.97
	Average	485	3.12	—	56	71	127	254		1.04
	1	200	2.46	nd	82	48	130	6	44	1.46
	2	199	2.63	nd	87	71	158	6	60	1.53
$PM_{2.5}$	3	224	2.32	nd	85	59	144	49	46	2.13
roadside #2	4	232	1.84	nd	70	63	133	13	81	0.95
	Average	214	2.31	—	81	61	142	19	58	1.52

Abbreviations: PM – particulate matter; DEE – diesel engine exhaust; GMD - geometric mean diameter; VOC – volatile organic components
†Aerodynamic particle size measured by MOI
‡Unreliable outcomes due to overload carbon samplers, those values are not included in the average VOC content

Bronchoalveolar lavage fluid analyses

Prolonged exposure to PM_{DEE} or $PM_{2.5}$ near a roadside did not induce a detectable inflammatory response in healthy rats. The number of MN in BALF was not significantly increased after 4 weeks of exposure to $PM_{2.5}$ roadside or PM_{DEE}; nor were there any changes in the pro-inflammatory cytokines TNF-α and IL-6. Although some parameters (e.g. TNF-α, protein) showed strong differences with higher values after exposure to roadside $PM_{2.5}$, the only statistically significant change was an increase in BALF CC16 after exposure to $PM_{2.5}$ in the second roadside study (9.47 ± 1.14 versus 8.49 ± 1.21 in the control group; $p<0.05$).

Notably, in particular protein and albumin levels in BALF were significantly higher in all animals that were transported to our field location near the freeway compared to those that were exposed in our laboratory at the RIVM (data not shown). Apart from the fact that batch-to-batch variation among the groups of animals that we received from the breeder cannot be excluded, the only other explanation is that the transport from the field location to the lab

might have resulted in increased stress and increased baseline values of the noted parameters. Since most of the other parameters that we have assessed did not to be affected in a similar manner, and we performed the (statistical) comparisons only within each of the three experiments, conclusions were not affected by this unexpected phenomenon.

Hematological analyses

Prolonged PM_{DEE} exposure resulted in significantly reduced numbers of white blood cells (WBC), lymphocytes and basophilic granulocytes (Table 3.2). On the other hand, neither $PM_{2.5}$ roadside exposure induced any significant changes in blood parameters, although a small decrease of lymphocyte number was observed in the second $PM_{2.5}$ roadside study. A reduction in the blood vWF levels was observed 4-weeks after exposure to PM_{DEE} (112.2 ± 34.2 versus 132.5 ± 13.2 mU/mL in the control group; $p<0.05$; Table 3.2).

Table 3.2. *Parameters in blood after exposure to diesel engine exhaust or concentrated ambient particles near a busy roadside*

		PM_{DEE}		$PM_{2.5}$ roadside #1		$PM_{2.5}$ roadside #2	
BALF		Control	PM exposure	Control	PM exposure	Control	PM exposure
Parameter	Unit	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
4 Weeks							
RBC	x 10^{12}/L	8.73 ± 0.36	8.65 ± 0.23	8.62 ± 0.23	8.59 ± 0.28	8.37 ± 0.20	8.41 ± 0.30
HGB	mmol/L	9.20 ± 0.39	9.02 ± 0.23	8.90 ± 0.31	8.93 ± 0.32	8.81 ± 0.21	8.82 ± 0.30
HCT	L/L	0.416 ± 0.016	0.412 ± 0.015	0.395 ± 0.015	0.396 ± 0.015	0.392 ± 0.010	0.393 ± 0.018
HDW	mmol/L	1.832 ± 0.079	1.855 ± 0.052	1.954 ± 0.071	1.930 ± 0.111	1.795 ± 0.101	1.806 ± 0.111
PLT	x 10^9/L	448 ± 57	431 ± 43	476 ± 89	462 ± 158	640 ± 60	671 ± 88
MPC	g/dL	22.59 ± 0.79	22.07 ± 0.93	22.87 ± 0.83	22.97 ± 0.75	22.81 ± 0.75	23.02 ± 0.61
WBC	x 10^9/L	3.69 ± 1.01	**2.91*±0.5**	3.34 ± 0.92	3.60 ± 1.11	4.23 ± 0.89	3.68 ± 0.82
PMN	x 10^9/L	0.77 ± 0.26	0.67 ± 0.18	0.76 ± 0.25	0.86 ± 0.28	0.68 ± 0.18	0.67 ± 0.18
Lymphocytes	x 10^9/L	2.78 ± 0.71	**2.14* ± 0.43**	2.47 ± 0.73	2.61 ± 0.85	3.36 ± 0.68	**2.85†± 0.64**
Basophils	x 10^9/L	0.008 ± 0.005	**0.002* ± 0.003**	0.026 ± 0.013	0.028 ± 0.017	0.026 ± 0.019	0.022 ± 0.007
PMN	%	20.6 ± 2.1	23.1 ± 5.6	23.1 ± 5.2	24.2 ± 5.7	16.2 ± 2.6	18.14 ± 2.74
Lymphocytes	%	75.86 ± 2.55	73.37 ± 5.79	74.02 ± 5.11	72.44 ± 6.11	79.58 ± 3.31	77.49 ± 2.79
Basophils	%	0.196 ± 0.069	0.132 ± 0.078	0.746 ± 0.263	0.778 ± 0.297	0.549 ± 0.307	0.583 ± 0.137
vWF	mU/mL	133 ± 13	**112* ± 34**	136 ± 29	124 ± 41	163 ± 22	153 ± 26
PAI-1	ng/mL	0.15 ± 0.12	0.24 ± 0.15	0.16 ± 0.1	0.22 ± 0.11	0.16 ± 0.13	0.20 ± 0.19
tPA tot	ng/mL	0.13 ± 0.04	0.12 ± 0.04	0.13 ± 0.05	0.13 ± 0.04		
Fibrinogen	mg/mL	1.74 ± 0.79	1.77 ± 0.67	1.52 ± 0.49	1.46 ± 0.65	2.63 ± 0.50	2.99 ± 1.16
CC16	ng/mL	30.3 ± 21.1	25.3 ± 14.1	19.5 ± 13.2	15.6 ± 10.1	25.9 ± 2.7	27.0 ± 4.5
Day 6							
vWF	mU/mL	193 ± 83	165 ± 78	198 ± 44	222 ± 21	137 ± 20	120 ± 37
PAI-1	ng/mL			0.27 ± 0.09	0.21 ± 0.12	0.37 ± 0.17	0.26 ± 0.19
CC16	ng/mL					25.1 ± 2.8	26.4 ± 4.3
Fibrinogen	mg/mL	1.24 ± 0.48	1.27 ± 0.76	1.05 ± 0.33	0.99 ± 0.26	2.44 ± 0.45	2.69 ± 0.71

*P<0.05 compared to experimental control; †P=0.05 compared to experimental control.

Lung pathology

The lungs of the animals exposed to PM_{DEE} showed a number of minor changes including perivascular and peribronchial inflammatory cell infiltrates, existing of mononuclear inflammatory cells (lymphocytes). The number of alveolar macrophages was generally low and there was no infiltration of neutrophilic or eosinophilic leukocytes. Although the incidence of a few changes was slightly increased, there were no changes that distinctly and convincingly could be related to PM_{DEE} exposure.

Roadside $PM_{2.5}$ exposures resulted in a diffuse accumulation of alveolar macrophages in the lungs of all animals, albeit in low numbers. There was no infiltration of neutrophilic or eosinophilic leukocytes. Diffuse macrophage accumulation tended to be slightly more severe in $PM_{2.5}$ exposed rats compared to rats exposed to filtered air (though not statistically significant). Alveolar macrophages of $PM_{2.5}$ exposed rats contained small dark-stained phagocytized particles, which were not observed in controls and should be therefore considered as a result of the PM exposure. Focal subpleural accumulations of alveolar macrophages accompanied by thickened alveolar septa occurred in animals exposed to all three PM test atmospheres. However, the incidence was significantly increased in roadside $PM_{2.5}$ exposed rats ($p<0.05$, Fisher's exact test). Since no adverse, treatment related effects were detected, no actual data on the pathological analysis are presented here.

Vascular function

In isolated rat aortic rings, the vasodilator PE caused a concentration-dependent contraction (Fig. 3.2). The response to PE was not different between control animals and PM_{DEE} exposed animals or control animals and animals exposed to roadside $PM_{2.5}$ ($p>0.05$ for all, two-way ANOVA; n=4-6). ACh, SNP and ISP all caused concentration-dependent relaxation of PE-contracted tissue. Responses in tissue from PM_{DEE} exposed animals or roadside $PM_{2.5}$ exposed animals were not different from their respective controls ($p>0.05$ for all). In light of these results, organ bath analysis was not performed in the repetition of the $PM_{2.5}$ roadside study.

Cardiac tissue

Samples of heart tissue from control and exposed rats were examined with regard to expression of IL-1β, TNF-α and IL-6 mRNA as well as to phosphorylation of MAPKs. Neither the expression of mRNA for these cytokines, nor the phosphorylation of the investigated MAPKs differed between control and PM-exposed animals (data not shown).

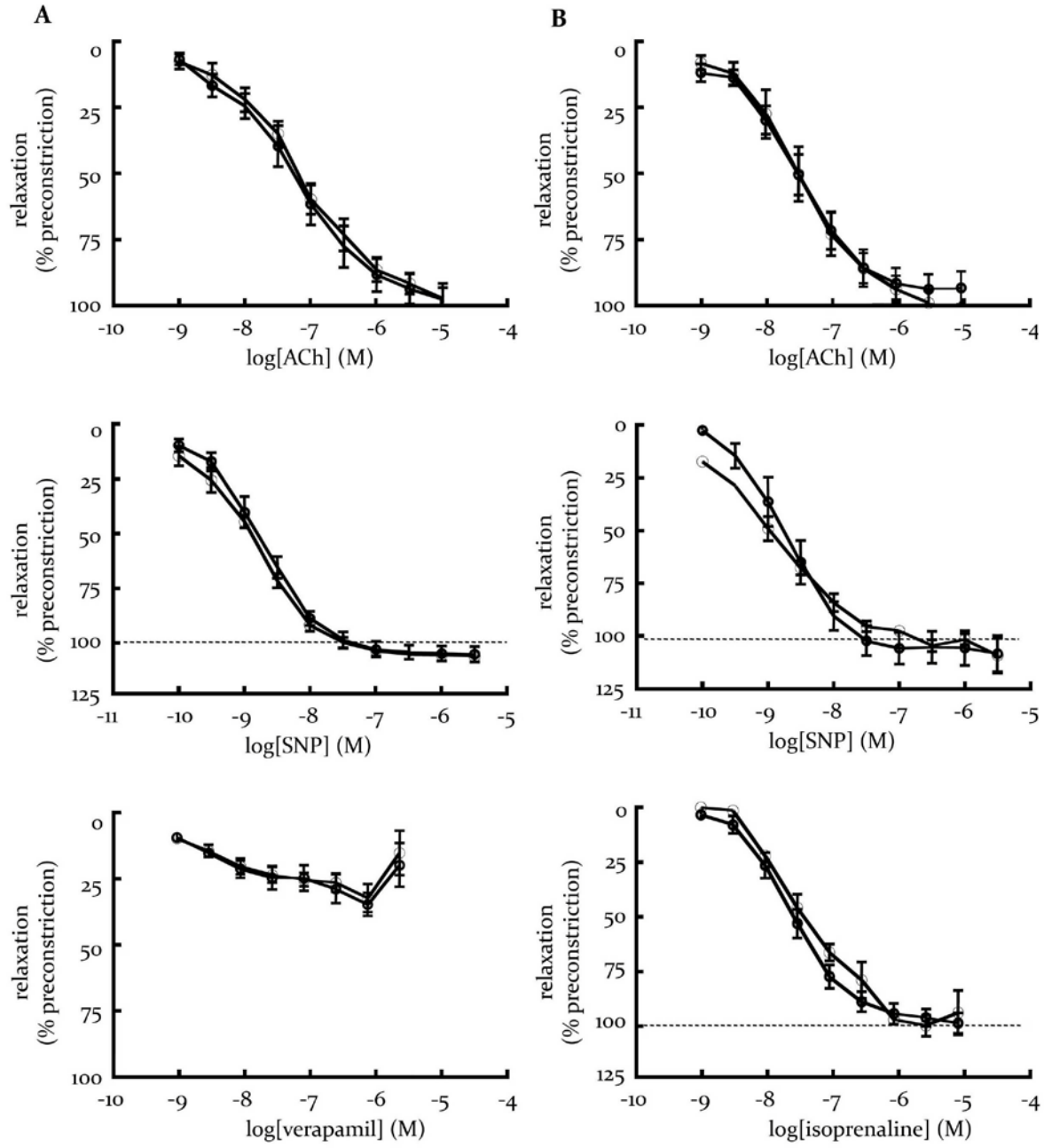


Figure 3.2. Effect of 4-week exposure to (A) diesel engine exhaust (PMDEE) or (B) concentrated ambient particles near a busy roadside (PM2.5 roadside) on *exvivo* responses to vasodilator agents in rat aortic rings. Exposed groups (filled circles) and filtered air control (open circles). Values are shown as mean ± SE(ACh, n = 5–6; SNP, n = 4–6; isoprenaline/verapamil, n = 4–6). There were no significant differences between PM and control exposures ($P > 0.05$ for all).

Tissue factor activity and thrombin generation

Lung tissue factor (TF) activity was significantly decreased after exposure to PM_{DEE} (control 368 ± 61 pM versus PM_{DEE} 218 ± 51 pM; p=0.009; Figure 3) and slightly diminished after $PM_{2.5}$ roadside exposure (control 348 ± 19 pM versus $PM_{2.5}$ 310 ± 34 pM; p=0.047; Fig. 3.3). Furthermore, partly coherent changes to lung TF activity were observed in thrombin generation, since the lag time is prolonged for exposure to PM_{DEE} (control 86% ± 3% versus PM_{DEE} 90% ± 3%; p=0.039; Fig. 3.3) while the lag time is shortened by exposure to $PM_{2.5}$ (control 97% ± 2% versus $PM_{2.5}$ roadside 91% ± 3%; p=0.015; Fig. 3.3) for the latter measured both with and without the addition of TF and phospholipids. Overall thrombin generation, as depicted by the ETP, was not altered upon long-term exposure to traffic-related PM: ETP control 51% ± 4% versus PM_{DEE} 46% ± 3% (p=0.231; Fig. 3.3) and control 58% ± 3% versus $PM_{2.5}$ roadside 51% ± 5% (p=0.383; Fig. 3.3). In addition, analysis of lung tissue thrombogenicity in the absence of additional TF and phospholipids demonstrated an overall decreased lung induced thrombin generation for long-term exposure to PM_{DEE} (ETP: control 38% ± 4% versus PM_{DEE} 28% ± 3%; p=0.027), whereas no changes were observed after long-term exposure to $PM_{2.5}$ (38% ± 3% versus $PM_{2.5}$ roadside 32% ± 3%; p=0.197). The attenuation of lung tissue induced thrombin generation upon exposure to PM_{DEE} was confirmed by a decrease in peak height (control 55% ± 6% versus PM_{DEE} 39% ± 4%; p=0.018; Fig. 3.3). Furthermore, maximum thrombin generation given by the peak height was decreased after $PM_{2.5}$ exposure (control 57% ± 5% versus $PM_{2.5}$ roadside 41% ± 5%; p=0.020; Fig. 3.3) confirming the trend in attenuation of the ETP.

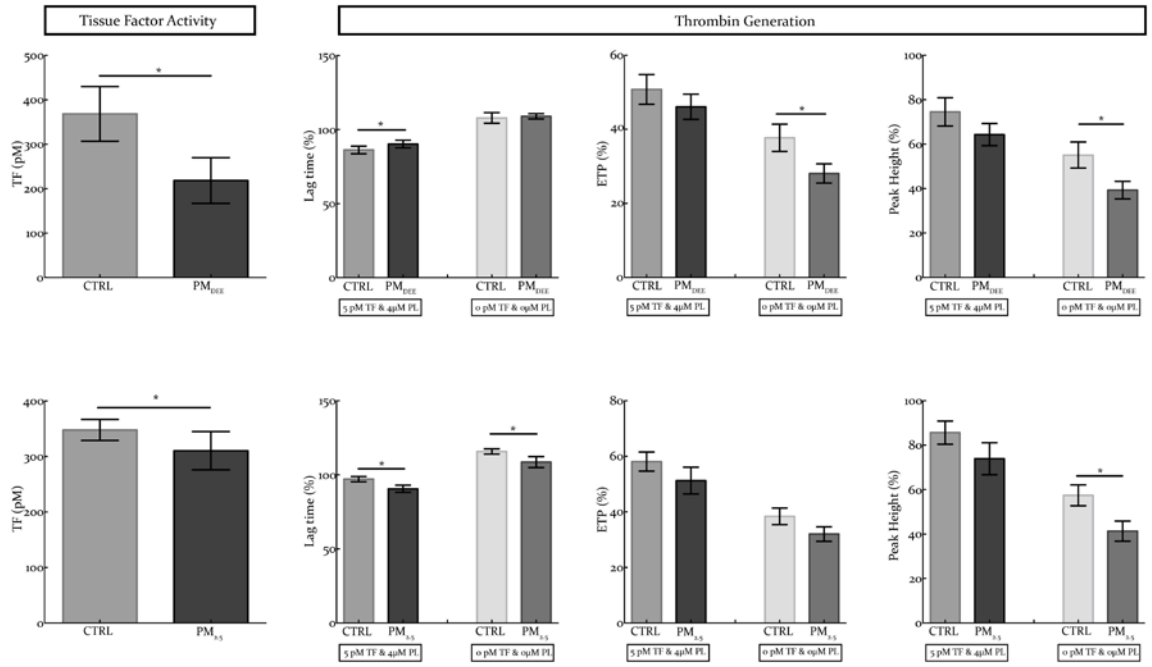


Figure 3.3. Tissue Factor (TF) activity in and overall procoagulant activity of lung homogenate after 4-weeks exposure to diesel engine exhaust (PM_{DEE}: upper panels) or concentrated ambient particles near a busy roadside ($PM_{2.5}$ roadside: lower panels). Lung tissue specific thrombin generation was performed in the presence of additional 5 pM TF and 4 µM phospholipids (5 pM TF & 4 µM PL) or in the absence of both TF and phospholipids (0 pM TF & 4 µM PL). Three parameters were derived from the obtained thrombin generation curves: lag time, defined as the time reaching 1/6 of the maximum peak thrombin; ETP, the endogenous thrombin potential or the area under the curve; and peak height, the maximum thrombin generated. Tissue Factor activity is expressed as pM corrected for total protein content of 2.5 mg/mL in the lung homogenate. Thrombin generation parameters are expressed as percentage of normal human pooled platelet poor plasma, which served as an internal control. Bars indicate mean ± SEM of n = 15 animals per groups. *$p < 0.05$.

Discussion

Prolonged exposure to traffic-related PM at levels approximately 10 times higher than ambient levels, or exposure to specifically diesel engine exhaust, exerted only modest effects in relatively healthy rats. This was irrespective the fact that a mild inflammation was induced at the onset of exposure. Accumulation of particles within alveolar macrophages was observed in both PM2.5 roadside exposures demonstrating that fine particulates are capable of reaching deep into the alveolar spaces. Biological changes were mainly of a cardiovascular nature, as shown by reduced WBC numbers, diminished levels of vWF protein, and reduced lung tissue thrombogenicity or procoagulant activity.

The fact that only very mild effects were detected in this study may be related to the adequately functioning host defense system of the rats. The animals were exposed to ozone (800 μg/m3 for 12 h) prior to prolonged exposure to traffic PM, which was intended to cause significant, yet nonsevere, pulmonary inflammation to compromise the defense system at the beginning of exposure to PM. Ozone is known to provoke damage of type I epithelial cells and increased permeability of the alveolar walls [45, 46]. Previous studies in our laboratory [47, 48] under similar conditions as in the current study (12–24 h; 800 μg/m3 ozone) resulted in a 2–3-fold increase protein levels in BALF, as well as a moderate influx of neutrophils (10–20% of total lavage cells). However, the inflammation induced in our study was rather mild, as only a slight (2.5%) increase in inflammatory cells was observed. On the other hand, a similar rise in lung permeability was found as reported previously. The difference in response might be caused by a difference in sensitivity between Fisher-344 rats used in the present study and the Wistar rats used previously. Nevertheless, ozone exposure was found to cause a similar degree of lung permeability to that found previously. This is important as an increase in permeability of the alveolar wall may assist in the translocation of particles from the lung into the circulation; one of the key mechanisms proposed to explain the systemic actions of inhaled particles [49]. Because accumulated particles within macrophages were observed in the present study, it seems plausible that translocation to the system circulation had taken place. Many epidemiological studies have claimed that, in particular, people with compromised airways are more likely to develop adverse health effects due to exposure to PM. This is in line with our observation that the rather healthy rats do not develop biological relevant adverse responses due to traffic-derived PM.

Using our diesel powered generator, a stable highly controlled test atmosphere was created that consisted of soot particles. The PM levels that were applied in this study can easily be detected in hot spots, such as road tunnels or at kerb sides of busy city streets. Ambient PM has been shown to have substantial spatial and temporal variation, both in terms of amount and physicochemical composition and that the contribution of secondary inorganic components although to play a very limited role in inducing toxicity [50] contributed on average ~25%. These factors might very well explain the intra- and inter-experimental variability observed for the two roadside experiments. The PM mass concentrations were higher than those applied in the PM_{DEE} exposure. On the basis of epidemiological associations that suggest a linear concentration–response relationship between PM and cardiorespiratory responses, it was assumed that PM mass concentrations would be predictive of the biological responses in the present study, however, this did not appear to be the case. Because the two roadside experiments led to higher, albeit distinctly different, average PM mass concentrations, according to the general assumption, any effect seen for PM_{DEE} should also be observed in the PM2.5 roadside experiments. However, most parameters responding in the PM_{DEE} experiment were not affected by the PM roadside exposures, which implies that other factors than PM mass (i.e. PM size and chemical composition) affect the *in vivo* responses. Indeed, previous studies by our group and others [11, 40, 51] suggested that factors such as chemical composition are driving the toxicity. Another important difference between the PM_{DEE} exposure and the exposure to roadside PM2.5 is the higher gaseous pollutant concentrations for the exposure to diesel engine exhaust. As the PM_{DEE} gaseous components were not exceeding limit values as defined by American Conference of Governmental Industrial Hygienists [52], we can assume that these could not explain the observed vascular responses.

One more variable between the PM_{DEE} and PM2.5 roadside exposures is the particle number concentration, with substantially higher numbers for the PM_{DEE} experiment. Diesel engine exhausts are dominated by particles of approximately <100 nm, which are also referred to as ultrafine particles. Several authors have suggested that ultrafine particles have adverse effects on the cardiovascular system [53-55]. Therefore, it may very well be that in our PM2.5 roadside experiments, in which the numbers of ultrafine particles were lower than the PM_{DEE}, the number of ultrafines has played a more dominant role than PM mass. Another explanation for the observed limited responses might be the development of adaptation caused by the long exposure duration. It is generally known that various biological markers have their optimal effect at different time points. Moreover, some markers like MAPKs may be activated over

time in a multi-phasic way, i.e. even baseline levels vary from day to day [56, 57]. Reduced vWF protein levels were already observed 6 days after exposure to PM_{DEE} and reached significance after 4 weeks. Measuring at the different time points might also implicate that changes in adaptative pathways are observed. This may explain some of the contradictory observations of other groups increased vWF levels to traffic-related PM [58, 59], compared to a decrease in vWF in association with air pollutants [60, 61]. Elevated plasma vWF levels may imply an increased risk for thrombosis [62], therefore, the time course of thrombotic responses may also vary between acute, subchronic, or prolonged exposure to air pollutants. Adaptation pathways to chronic exposures present in healthy animals may be impaired in disease; therefore, experiments are currently underway in our laboratory examining the actions of PM_{DEE} on the cardiovascular system in a model of atherosclerosis.

The impaired lung procoagulation activity after prolonged exposure to traffic-related PM is supported by the reduced lung TF activity in conjunction with reduced lung tissue thrombin generation. In general, acute exposure to air pollution is associated to increased hypercoagulability shown by shorter prothrombin time (lag time) and elevated plasma thrombin generation [2, 63]. However, these epidemiological studies provide insight in the plasma hypercoagulable state after acute exposure to air pollution, whereas impaired tissue procoagulant activity might also indicate an adaptive defense mechanism.

In our clinical studies, we have shown that a 2-h exposure of healthy volunteers to Edinburgh PM2.5 had no effect on vessel wall function as determined by forearm plethysmography [64]. On the other hand, diluted DEE attenuated responses to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP, but not to the NO-independent vasodilator, verapamil. Previously, we have demonstrated in an animal experiment that similar responses occurred 4 h after acute exposure to various PM samples by intratracheal instillation [41]. In addition, diesel engine exhaust particles directly inhibit vascular relaxation to endothelium dependent vasodilators [42]. Therefore, we assumed that prolonged exposure to PM_{DEE} and not roadside PM2.5 was associated with cardiovascular impairment. However, no signs of impairment were observed after prolonged exposures applied in the three experiments presented in this paper. In the present study, we noted a decrease of WBC in the PM_{DEE} as well as in the second PM2.5 roadside experiment. Similar observations have been made in rats after acute exposure to traffic-related PM [27, 39]. In human studies, Frampton and co-workers noted that NO2 exposure resulted in reduced lymphocytes that migrate to the lung, as increased lymphocyte numbers were found in the

respiratory system [65]. Recently, changes in differential WBC was reported in patients with chronic pulmonary disease related to ambient air pollution exposure [66]. Although the biological significance and impact is still not clear, it seems that reduced circulating WBCs are related to increased exposure to air pollutants.

Freshly generated PM_{DEE} induced mild cardiovascular responses (impaired coagulation) but no respiratory effects were seen in relatively healthy rats. Also no biological relevant changes were detected after exposure to ambient roadside PM2.5. The overall analysis of the results did not support the hypothesis that PM mass concentrations are linear related to health effects. In contrast to common belief, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms. It could be speculated that vulnerability of humans to acute or repeated exposure to PM may be primarily dependent on the presence of comorbidity such as coronary heart disease. We conclude that prolonged although not chronic exposures in healthy animals have very limited impact on pulmonary and cardiovascular function. Further studies are needed in animals with established disease (such as more extensive pulmonary inflammation or developed cardiovascular disease) to reveal the influence of susceptibility on air pollution induced toxicity.

Acknowledgements

We thank the technical team under the supervision of Ruud van Kinderen and Hans Strootman from the Animal Facility Department of the National Institute of Vaccines for their skillful biotechnical assistance. We also acknowledge the assistants, Liset de la Fonteyne-Blankestijn, Yvonne Wallbrink, Piet Beekhof, and colleagues under the supervision of Henk van Loveren and Eugene Jansen, from the Laboratory for Health Protection Research of the RIVM's Nutrition, Medicines and Consumer Safety Division for their excellent technical support.

Declarations of interest

The main part of this work was supported by a fund from the Dutch Ministry of Housing, Spatial Planning and the Environment (VROM) in the framework of research project Air Pollution and Health (M/630186 and M/630196) at the RIVM. Mark Miller and Patrick Hadoke are supported by a British Heart Foundation Programme Grant (RG/05/003). Evren Kilinç is supported by a grant of the Netherlands Heart Foundation (grant number: 2006B064). The analyses of heart tissues conducted at the Norwegian Institute of Public Health were funded by the Research Council of Norway through the "Environment, Genetics and Health" program. This work has been presented in part at the Inhaled Particles X Conference 2008 and published in the accompanying *Journal of Physics Conference Series* 2009.

References

1 Brunekreef B, Holgate ST. Air pollution and health. *Lancet*. 2002; **360**: 1233-42. 10.

2 Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost*. 2007; **5**: 252-60.

3 Rudez G, Janssen NA, Kilinc E, Leebeek FW, Gerlofs-Nijland ME, Spronk HM, ten Cate H, Cassee FR, de Maat MP. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect*. 2009; **117**: 995-1001.

4 Pope CA, 3rd, Burnett RT, Thurston GD, Thun MJ, Calle EE, Krewski D, Godleski JJ. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation*. 2004; **109**: 71-7.

5 Schikowski T, Sugiri D, Ranft U, Gehring U, Heinrich J, Wichmann HE, Kramer U. Long-term air pollution exposure and living close to busy roads are associated with COPD in women. *Respir Res*. 2005; **6**: 152.

6 Gehring U, Heinrich J, Kramer U, Grote V, Hochadel M, Sugiri D, Kraft M, Rauchfuss K, Eberwein HG, Wichmann HE. Long-term exposure to ambient air pollution and cardiopulmonary mortality in women. *Epidemiology*. 2006; **17**: 545-51.

7 Hoek G, Brunekreef B, Goldbohm S, Fischer P, van den Brandt PA. Association between mortality and indicators of traffic-related air pollution in the Netherlands: a cohort study. *Lancet*. 2002; **360**: 1203-9.

8 Beelen R, Hoek G, Houthuijs D, van den Brandt PA, Goldbohm RA, Fischer P, Schouten LJ, Armstrong B, Brunekreef B. The joint association of air pollution and noise from road traffic with cardiovascular mortality in a cohort study. *Occup Environ Med*. 2009; **66**: 243-50.

9 Gauderman WJ, Avol E, Lurmann F, Kuenzli N, Gilliland F, Peters J, McConnell R. Childhood asthma and exposure to traffic and nitrogen dioxide. *Epidemiology*. 2005; **16**: 737-43.

10 Hoffmann B, Moebus S, Dragano N, Mohlenkamp S, Memmesheimer M, Erbel R, Jockel KH. Residential traffic exposure and coronary heart disease: results from the Heinz Nixdorf Recall Study. *Biomarkers*. 2009; **14 Suppl 1**: 74-8.

11 Gerlofs-Nijland ME, Dormans JA, Bloemen HJ, Leseman DL, John A, Boere F, Kelly FJ, Mudway IS, Jimenez AA, Donaldson K, Guastadisegni C, Janssen NA, Brunekreef B, Sandstrom T, van Bree L, Cassee FR. Toxicity of coarse and fine particulate matter from sites with contrasting traffic profiles. *Inhal Toxicol*. 2007; **19**: 1055-69.

12 Lai CH, Liou SH, Lin HC, Shih TS, Tsai PJ, Chen JS, Yang T, Jaakkola JJ, Strickland PT. Exposure to traffic exhausts and oxidative DNA damage. *Occup Environ Med*. 2005; **62**: 216-22.

13 Seagrave J, McDonald JD, Bedrick E, Edgerton ES, Gigliotti AP, Jansen JJ, Ke L, Naeher LP, Seilkop SK, Zheng M, Mauderly JL. Lung toxicity of ambient particulate matter from southeastern U.S. sites with different contributing sources: relationships between composition and effects. *Environ Health Perspect*. 2006; **114**: 1387-93.

14 Elder A, Couderc JP, Gelein R, Eberly S, Cox C, Xia X, Zareba W, Hopke P, Watts W, Kittelson D, Frampton M, Utell M, Oberdorster G. Effects of on-road highway aerosol exposures on autonomic responses in aged, spontaneously hypertensive rats. *Inhal Toxicol*. 2007; **19**: 1-12.

15 McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, Harrington R, Svartengren M, Han IK, Ohman-Strickland P, Chung KF, Zhang J. Respiratory effects of exposure to diesel traffic in persons with asthma. *N Engl J Med*. 2007; **357**: 2348-58.

16 Mills NL, Tornqvist H, Gonzalez MC, Vink E, Robinson SD, Soderberg S, Boon NA, Donaldson K, Sandstrom T, Blomberg A, Newby DE. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N Engl J Med*. 2007; **357**: 1075-82.

17 Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandstrom T, Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation*. 2005; **112**: 3930-6.

18 Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. Diesel exhaust inhalation increases thrombus formation in man. *Eur Heart J*. 2008; **29**: 3043-51.

19 Kim S, Jaques PA, Chang MC, Barone T, Xiong C, Friedlander SK, Sioutas C. Versatile aerosol concentration enrichment system (VACES) for simultaneous in vivo and in vitro evaluation of toxic effects of ultrafine, fine and coarse ambient particles - Part II: Field evaluation. *J Aerosol Sci*. 2001; **32**: 1299-314.

20 Kim S, Jaques PA, Chang MC, Froines JR, Sioutas C. Versatile aerosol concentration enrichment system (VACES) for simultaneous in vivo and in vitro evaluation of toxic effects of ultrafine, fine and coarse ambient particles - Part I: Development and laboratory characterization. *J Aerosol Sci*. 2001; **32**: 1281-97.

21 Sioutas C, Koutrakis P, Godleski JJ, Ferguson ST, Kim CS, Burton RM. Fine particle concentrators for inhalation exposures - Effect of particle size and composition. *J Aerosol Sci*. 1997; **28**: 1057-71.

22 Lippmann M, Chen LC. Health effects of concentrated ambient air particulate matter (CAPs) and its components. *Crit Rev Toxicol*. 2009; **39**: 865-913.

23 Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ, Nel AE. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circ Res*. 2008; **102**: 589-96.

24 Cassee FR, Boere AJ, Fokkens PH, Leseman DL, Sioutas C, Kooter IM, Dormans JA. Inhalation of concentrated particulate matter produces pulmonary inflammation and systemic biological effects in compromised rats. *J Toxicol Environ Health A*. 2005; **68**: 773-96.

25 Gong H, Jr., Sioutas C, Linn WS. Controlled exposures of healthy and asthmatic volunteers to concentrated ambient particles in metropolitan Los Angeles. *Res Rep Health Eff Inst*. 2003: 1-36; discussion 7-47.

26 Kleinman MT, Hamade A, Meacher D, Oldham M, Sioutas C, Chakrabarti B, Stram D, Froines JR, Cho AK. Inhalation of concentrated ambient particulate matter near a heavily trafficked road stimulates antigen-induced airway responses in mice. *J Air Waste Manag Assoc*. 2005; **55**: 1277-88.

27 Kooter IM, Boere AJ, Fokkens PH, Leseman DL, Dormans JA, Cassee FR. Response of spontaneously hypertensive rats to inhalation of fine and ultrafine particles from traffic: experimental controlled study. *Part Fibre Toxicol*. 2006; **3**: 7. 10.1186/1743-8977-3-7.

28 Lippmann M, Hwang JS, Maciejczyk P, Chen LC. PM source apportionment for short-term cardiac function changes in ApoE-/- mice. *Environ Health Perspect*. 2005; **113**: 1575-9.

29 Ying Z, Kampfrath T, Thurston G, Farrar B, Lippmann M, Wang A, Sun Q, Chen LC, Rajagopalan S. Ambient particulates alter vascular function through induction of reactive oxygen and nitrogen species. *Toxicol Sci*. 2009; **111**: 80-8.

30 Chen LC, Hwang JS. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. IV. Characterization of acute and chronic effects of ambient air fine particulate matter exposures on heart-rate variability. *Inhal Toxicol*. 2005; **17**: 209-16.

31 Chen LC, Nadziejko C. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. V. CAPs exacerbate aortic plaque development in hyperlipidemic mice. *Inhal Toxicol*. 2005; **17**: 217-24.

32 Hwang JS, Nadziejko C, Chen LC. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. III. Acute and chronic effects of CAPs on heart rate, heart-rate fluctuation, and body temperature. *Inhal Toxicol*. 2005; **17**: 199-207.

33 Lippmann M, Gordon T, Chen LC. Effects of subchronic exposures to concentrated ambient particles in mice. IX. Integral assessment and human health implications of subchronic exposures of mice to CAPs. *Inhal Toxicol*. 2005; **17**: 255-61.

34 Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *Jama*. 2005; **294**: 3003-10.

35 Sun Q, Yue P, Kirk RI, Wang A, Moatti D, Jin X, Lu B, Schecter AD, Lippmann M, Gordon T, Chen LC, Rajagopalan S. Ambient air particulate matter exposure and tissue factor expression in atherosclerosis. *Inhal Toxicol*. 2008; **20**: 127-37.

36 Veronesi B, Makwana O, Pooler M, Chen LC. Effects of subchronic exposures to concentrated ambient particles. VII. Degeneration of dopaminergic neurons in Apo E-/- mice. *Inhal Toxicol*. 2005; **17**: 235-41.

37 Gerlofs-Nijland ME, Campbell A, Miller MR, Newby DE, Cassee FR. Toxicity of inhaled traffic related particulate matter. *J Phys: Conf Ser*. 2009; **151** 012049.

38 Marra M, Rombout PJA. Design and performance of an inhalation chamber for exposing laboratory animals to oxidant air pollutants. *Inhal Toxicol*. 1990; **2**: 187-204.

39 Gerlofs-Nijland ME, Boere AJ, Leseman DL, Dormans JA, Sandstrom T, Salonen RO, van Bree L, Cassee FR. Effects of particulate matter on the pulmonary and vascular system: time course in spontaneously hypertensive rats. *Part Fibre Toxicol*. 2005; **2**: 2.

40 Gerlofs-Nijland ME, Rummelhard M, Boere AJ, Leseman DL, Duffin R, Schins RP, Borm PJ, Sillanpaa M, Salonen RO, Cassee FR. Particle induced toxicity in relation to transition metal and polycyclic aromatic hydrocarbon contents. *Environ Sci Technol*. 2009; **43**: 4729-36.

41 Bagate K, Meiring JJ, Gerlofs-Nijland ME, Vincent R, Cassee FR, Borm PJ. Vascular effects of ambient particulate matter instillation in spontaneous hypertensive rats. *Toxicol Appl Pharmacol*. 2004; **197**: 29-39.

42 Miller MR, Borthwick SJ, Shaw CA, McLean SG, McClure D, Mills NL, Duffin R, Donaldson K, Megson IL, Hadoke PW, Newby DE. Direct impairment of vascular function by diesel exhaust particulate through reduced bioavailability of endothelium-derived nitric oxide induced by superoxide free radicals. *Environ Health Perspect*. 2009; **117**: 611-6.

43 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.

44 Spronk HM, Dielis AW, De Smedt E, van Oerle R, Fens D, Prins MH, Hamulyak K, ten Cate H. Assessment of thrombin generation II: Validation of the Calibrated Automated Thrombogram in platelet-poor plasma in a clinical laboratory. *Thromb Haemost*. 2008; **100**: 362-4.

45 Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev*. 1999; **2**: 31-86.

46 Dormans JA, Rombout PJ, van Loveren H. Surface morphology and morphometry of rat alveolar macrophages after ozone exposure. *J Toxicol Environ Health*. 1990; **31**: 53-70.

47 van Bree L, Dormans JA, Boere AJ, Rombout PJ. Time study on development and repair of lung injury following ozone exposure in rats. *Inhal Toxicol*. 2001; **13**: 703-18.

48 van Bree L, Dormans JA, Koren HS, Devlin RB, Rombout PJ. Attenuation and recovery of pulmonary injury in rats following short-term, repeated daily exposure to ozone. *Inhal Toxicol*. 2002; **14**: 883-900.

49 Geiser M, Kreyling WG. Deposition and biokinetics of inhaled nanoparticles. *Part Fibre Toxicol*. 2010; **7**: 2.

50 Schlesinger RB, Cassee F. Atmospheric secondary inorganic particulate matter: the toxicological perspective as a basis for health effects risk assessment. *Inhal Toxicol*. 2003; **15**: 197-235.

51 Schwarze PE, Ovrevik J, Lag M, Refsnes M, Nafstad P, Hetland RB, Dybing E. Particulate matter properties and health effects: consistency of epidemiological and toxicological studies. *Hum Exp Toxicol*. 2006; **25**: 559-79.

52 ACGIH. Documentation of the threshold limit values and biological exposure indices. 6th edn. Cincinnati, OH: American Conference of Governmental Industrial Hygienists, 1991.

53 Delfino RJ, Sioutas C, Malik S. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. *Environ Health Perspect*. 2005; **113**: 934-46.

54 Knol AB, de Hartog JJ, Boogaard H, Slottje P, van der Sluijs JP, Lebret E, Cassee FR, Wardekker JA, Ayres JG, Borm PJ, Brunekreef B, Donaldson K, Forastiere F, Holgate ST, Kreyling WG, Nemery B, Pekkanen J, Stone V, Wichmann HE, Hoek G. Expert elicitation on ultrafine particles: likelihood of health effects and causal pathways. *Part Fibre Toxicol*. 2009; **6**: 19.

55 Schulz H, Harder V, Ibald-Mulli A, Khandoga A, Koenig W, Krombach F, Radykewicz R, Stampfl A, Thorand B, Peters A. Cardiovascular effects of fine and ultrafine particles. *J Aerosol Med*. 2005; **18**: 1-22.

56 Chen JX, Berry LC, Christman BW, Meyrick B. Glutathione mediates LPS-stimulated COX-2 expression via early transient p42/44 MAPK activation. *J Cell Physiol*. 2003; **197**: 86-93.

57 Thrane EV, Refsnes M, Thoresen GH, Lag M, Schwarze PE. Fluoride-induced apoptosis in epithelial lung cells involves activation of MAP kinases p38 and possibly JNK. *Toxicol Sci*. 2001; **61**: 83-91.

58 O'Neill MS, Veves A, Sarnat JA, Zanobetti A, Gold DR, Economides PA, Horton ES, Schwartz J. Air pollution and inflammation in type 2 diabetes: a mechanism for susceptibility. *Occup Environ Med*. 2007; **64**: 373-9.

59 Yue W, Schneider A, Stolzel M, Ruckerl R, Cyrys J, Pan X, Zareba W, Koenig W, Wichmann HE, Peters A. Ambient source-specific particles are associated with prolonged repolarization and increased levels of inflammation in male coronary artery disease patients. *Mutat Res*. 2007; **621**: 50-60.

60 Carlsten C, Kaufman JD, Trenga CA, Allen J, Peretz A, Sullivan JH. Thrombotic markers in metabolic syndrome subjects exposed to diesel exhaust. *Inhal Toxicol*. 2008; **20**: 917-21.

61 Hildebrandt K, Ruckerl R, Koenig W, Schneider A, Pitz M, Heinrich J, Marder V, Frampton M, Oberdorster G, Wichmann HE, Peters A. Short-term effects of air pollution: a panel study of blood markers in patients with chronic pulmonary disease. *Part Fibre Toxicol*. 2009; **6**: 25.

62 Franchini M, Mannucci PM. Von Willebrand factor: another janus-faced hemostasis protein. *Semin Thromb Hemost*. 2008; **34**: 663-9.

63 Bonzini M, Tripodi A, Artoni A, Tarantini L, Marinelli B, Bertazzi PA, Apostoli P, Baccarelli A. Effects of inhalable particulate matter on blood coagulation. *J Thromb Haemost*. 2010; **8**: 662-8.

64 Mills NL, Robinson SD, Fokkens PH, Leseman DL, Miller MR, Anderson D, Freney EJ, Heal MR, Donovan RJ, Blomberg A, Sandstrom T, MacNee W, Boon NA, Donaldson K, Newby DE, Cassee FR. Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease. *Environ Health Perspect*. 2008; **116**: 709-15.

65 Frampton MW, Boscia J, Roberts NJ, Jr., Azadniv M, Torres A, Cox C, Morrow PE, Nichols J, Chalupa D, Frasier LM, Gibb FR, Speers DM, Tsai Y, Utell MJ. Nitrogen dioxide exposure: effects on airway and blood cells. *Am J Physiol Lung Cell Mol Physiol*. 2002; **282**: L155-65.

66 Bruske I, Hampel R, Socher MM, Ruckerl R, Schneider A, Heinrich J, Oberdorster G, Wichmann HE, Peters A. Impact of ambient air pollution on the differential white blood cell count in patients with chronic pulmonary disease. *Inhal Toxicol*. 2010; **22**: 245-52.

CHAPTER 4

FACTOR XII ACTIVATION IS ESSENTIAL TO SUSTAIN THE PRO-COAGULANT EFFECTS OF PARTICULATE MATTER

Based on:

Kilinç, E., van Oerle, R., Borissoff, J.I., Oschatz, C., Gerlofs-Nijland, M.E., Janssen, N.A.H., Cassee, F.R., Sandström, T., Renné, T., ten Cate, H., Spronk, H.M.H.

2011. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *J. Thromb and Haemost.* 9(7):1359-1367.

Abstract

Particulate matter (PM) is a key component of ambient air pollution and has been associated with an increased risk of thrombotic events and mortality. The underlying mechanisms remain unclear.

We aimed to study the mechanisms of PM-driven procoagulant activity in human plasma and to investigate mainly, the ultrafine particles (UFPs; <0.1μm) driven coagulation in genetically modified mice.

Thrombin generation in response to PM of different sizes was assessed in normal human platelet poor plasma, as well as in plasmas deficient in the intrinsic pathway proteases factors XII (FXII) or XI (FXI). In addition, UFPs were intratracheally instilled in wild-type (WT) and factor XII-deficient ($FXII^{-/-}$) mice and plasma thrombin generation was analyzed in plasma from treated mice at 4 and 20 h post exposure.

In normal human plasma thrombin generation was enhanced in the presence of PM, whereas PM-driven thrombin formation was completely abolished in FXII- and FXI-deficient plasma. UFPs induced a transient increase in tissue factor (TF) driven thrombin formation at 4h post instillation in WT mice compared to saline instillation. Intratracheal instillation of UFPs resulted in a procoagulant response in WT mice plasma at 20 h, whereas it was entirely suppressed in $FXII^{-/-}$ mice.

Overall, the data suggest that PM promotes its early procoagulant actions mostly through TF-driven extrinsic pathway of coagulation, whereas PM-driven long lasting thrombogenic effects are predominantly mediated via formation of activated FXII. Hence, FXII-driven thrombin formation may be relevant to an enhanced thrombotic susceptibility upon chronic exposure to PM in human.

Introduction

Particulate matter (PM) comprises all small components of air pollution and is derived from both natural and anthropogenic sources such as wood smoke and engine exhaust [1]. PM can be classified in three main sub-types according to particle size: coarse between 2.5 and 10 μm; fine in the range between 0.15 and 2.5 μm, and ultrafine particles (UFPs) with a diameter smaller than 0.1 μm [2]. Epidemiological and experimental studies suggest that exposure to PM is associated with increased risk for arterial and venous thrombosis, and increased risk of cardiovascular death [1, 3-6].

Exposure to PM has been associated with a hypercoagulable state in both humans and rodents [7], although the mechanistic basis for PM triggered procoagulant activity is poorly understood. One possible mechanism for PM-driven procoagulant state may be the increased expression of tissue factor (TF) in lung tissue [8] through inflammatory mediators such as interleukin-6 [9]. In addition, suppressed endothelial thrombomodulin activity aggravates the procoagulant response to PM in rodents [8]. Moreover, inhalation of airborne PM in humans has been shown to result in pulmonary inflammation and systemic inflammation, reduced release of tissue-plasminogen activator (t-PA), increased activation and adhesiveness of platelets resulting in increased *ex vivo* thrombosis generation [10-12].

Results of animal exposure studies suggest that due to their small size, UFPs can transmigrate through the lung epithelium and vascular barrier into the systemic circulation [13-19], where they may directly interact with blood cells and plasma proteins. On the other hand, whether UFPs can readily access the circulation in humans is still controversial [20, 21].

In case of translocation into the plasma compartment, UFPs may interact with the intrinsic pathway of coagulation, which is initiated upon contact of factor XII (FXII) to negatively charged surfaces. In addition to the traditional concept where anionic surfaces activate FXII, FXII activation in plasma occurs with a greater efficiency at hydrophilic surfaces than at an equal surface area of hydrophobic ones [22]. Recently, physiological FXII activators such as collagen, misfolded proteins, and polyphosphates were identified [23-25]. Activated factor XII (FXIIa) drives the intrinsic pathway by activating its substrate factor XI (FXI), which in turn activates factor IX (FIX) [26], ultimately resulting in fibrin formation. Studies using FXII deficient ($FXII^{-/-}$) mice implicate a role for the intrinsic pathway of coagulation in arterial thrombosis [27], cerebral ischemia reperfusion injury [28] and pulmonary embolism [24]. Despite that the exact role of FXII in thrombosis remains to be further elucidated [29], clinical

evidence has shown positive association with an increased risk of acute thrombotic events [30, 31].

Hereby, we hypothesize that PM can enhance the formation of FXIIa, thereby inducing a hypercoagulable state. Our hypothesis was tested in human plasma for coarse, fine and UFPs collected from different locations and furthermore, in $FXII^{-/-}$ and wild-type (WT) mice, challenged with UFPs by intratracheal instillation.

Material and methods

Sampling and characterization of PM

Coarse and fine PM samples were collected at various European locations with contrasting traffic intensities (1: Hendrik Ido Ambacht roadside tunnel, 2: Dordrecht, the Netherlands and 3: München east train station, 4: München Grosshadern, Germany) on polyurethane foam (PUF) using a high-volume cascade impactor [32]. Additionally, coarse, fine + UFPs and UFPs biosamples were collected using a Versatile Aerosol Concentration Enrichment System (VACES) [33] equipped with Biosamplers (SKC Inc., Eighty-Four PA; coarse, fine + UFPs, and UFPs of PM) at a platform of an underground train station (Amsterdam, the Netherlands) at two different time points. The UFPs biosample which was administered in mice was collected with the same method near a Dutch roadside tunnel (Hendrik Ido Ambacht) that is mainly used by heavy diesel trucks.

Thrombin generation in human plasma

Plasma thrombin generation was measured by means of the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, the Netherlands) [34], which makes use of a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously monitor thrombin activity in clotting plasma. In order to correct for inner-filter effects and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed amount of thrombin-α2-macroglobulin complex (Thrombin Calibrator, Thrombinoscope BV), as recommended by the manufacturer. Fluorescence was read in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 filter set, and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV) as described previously [35].

The FXII mediated effect on thrombin generation *in vitro* of PM from different locations was first measured using coarse and fine PM collected on PUF while the effect of all three PM size

categories was examined using two biosamples from an underground train station according to the following protocol; 10 µL PM (at 4 different concentrations) in saline was applied into 80 µL normal human pooled platelet poor plasma, FXII- or FXI-deficient plasma (George King Biomedical, Overland Park, KS). Thrombin generation was recorded in the absence or presence of 4 µM phospholipids (Thrombinoscope BV) with PM solution at concentrations of 3.6, 1.8, 0.9, 0.45 and 0 µg/mL. Furthermore, the reaction was started upon addition of calcium and fluorogenic substrate. Three parameters were derived from the thrombin generation curves: lag time, peak height and endogenous thrombin potential (ETP, area under the curve).
Normal human pooled platelet poor plasma was prepared from 90 healthy volunteers not taking any medication. Venous blood was collected in 3.2% (w/v) citrate tubes using a 21-gauge needle (BD). The first 10 mL of venous blood were discarded. Platelet-poor plasma was prepared by two centrifugation steps: the first at 2,000x g for 15 minutes and the second at 11,000x g for 10 min. Obtained plasmas were pooled and aliquots were snap-frozen in liquid nitrogen and stored at -80 °C until use. All samples were thawed at 37 °C for 15 min before analysis.
Active side-inhibited factor VII (ASIS; kindly provided by Dr. L. Petersen, Novo Nordisk, Denmark) at 30 nM final concentration was added to human plasma in all *in vitro* thrombin generations in human plasma before analysis, in order to investigate the contribution of the intrinsic pathway of coagulation to thrombin generation in the presence of PM. Furthermore, corn trypsin inhibitor (CTI; Haematologic Technologies Inc., Essex Junction, VT) with a final concentration of 40 µg/mL was added to inhibit FXII activity in one of the experimental settings. The thrombin generation experiments with PM were also performed in the presence of phospholipids only (no ASIS), in order to determine the contribution of activation of the extrinsic pathway of coagulation by PM-derived tissue factor on thrombin generation.

Activation of FXII

Enzymatic FXIIa activity was determined from the cleavage of Pefachrome FXIIa substrate (DSM Nutritional products Ltd. Branch Pentapharm, Basel/ Switzerland) at 37°C. Incubations contained purified FXII (95 nM), prekallikrein (PK; 30 nM), and high-molecular-weight kininogen (HMWK; 30 nM) in Tris –Imidazol buffer pH 7.9 (150 mM NaCl, 50 mM Tris Base, 50 mM Imidazol) all at final concentrations, after addition of the substrate [25]. In order to investigate whether PM enhances FXII activation in the absence and presence of PK and HMWK, coarse PM (at 83.3; 41.6; 20.8; 10.4; 0 µg/ml) and purified FXII were preincubated for

20 minutes at 37 ˚C. After addition of 0.8 mM Pefachrome FXIIa substrate with and without PK and HMWK, the increase in absorption at 405 nm was determined (linear in time).

Animals and Intratracheal instillation of UFPs

Eight weeks old male $FXII^{-/-}$ mice [27, 28] and WT littermate mice were challenged with UFPs biosample (collected near a Dutch roadside tunnel; mainly used by heavy diesel trucks) suspended in sterilized saline at a concentration of 3.6 µg/mL. In addition, sterile saline was instilled in WT mice as control. Mice were anesthetized with 350 µL Avertin (25 µg/mL intraperitonally) and UFPs were instilled intratracheally in a volume of 100-120 µL per mouse. After instillation, mice were placed in the right and left lateral decubitus position for 10–15 seconds each site. Four and 20 h after instillation, blood samples were collected as described previously [36]. The protocol for the use of mice was approved by the Animal Care and Use Committee at Maastricht University.

Thrombin generation in mouse plasma

Thrombin generation in mouse plasma was recorded using 10 µL plasma diluted with 30 µL HEPES buffer (25 nM HEPES, 175 nM NaCl, pH 7.7) and 4 µM final concentration of phospholipids Additionally, thrombin generation in mouse plasma was assessed either in the presence of 40 µg/mL CTI and 30 nM ASIS in order to evaluate the contribution of FXIa to thrombin generation in plasma, only in the presence of 40 µg/mL CTI to measure TF, or in the presence of 30 nM ASIS to assess the contribution of FXIIa.

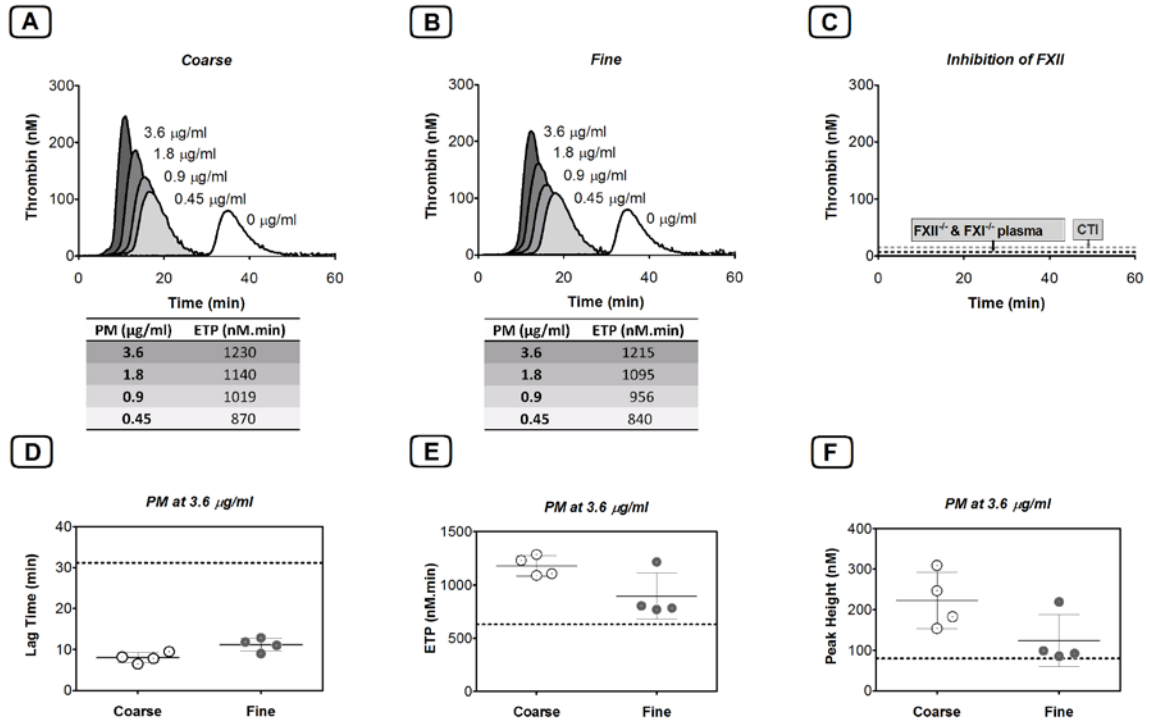
Statistical Analysis

Results are expressed as mean ± SD. Data analysis was performed by using Prism 5 for Windows, version 5.01 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were compared using the one-way ANOVA test with Bonferroni correction and statistical significance was defined as $p < 0.05$.

Results

PM enhances thrombin formation by the intrinsic pathway of coagulation in vitro

To study the contribution of the intrinsic pathway of coagulation to thrombin generation in the presence of PM in different size ranges and from various locations in human plasma, we analyzed PM-induced thrombin generation by means of CAT. All PM (coarse, fine) from different locations dose-dependently enhanced thrombin generation in normal human pooled platelet poor plasma (in the presence of ASIS) as indicated by a shortening in lag times, as well as by increased maximal (peak height) and total (ETP) thrombin formed compared to plasma thrombin generation in the absence of PM (Fig. 4.1A and B).



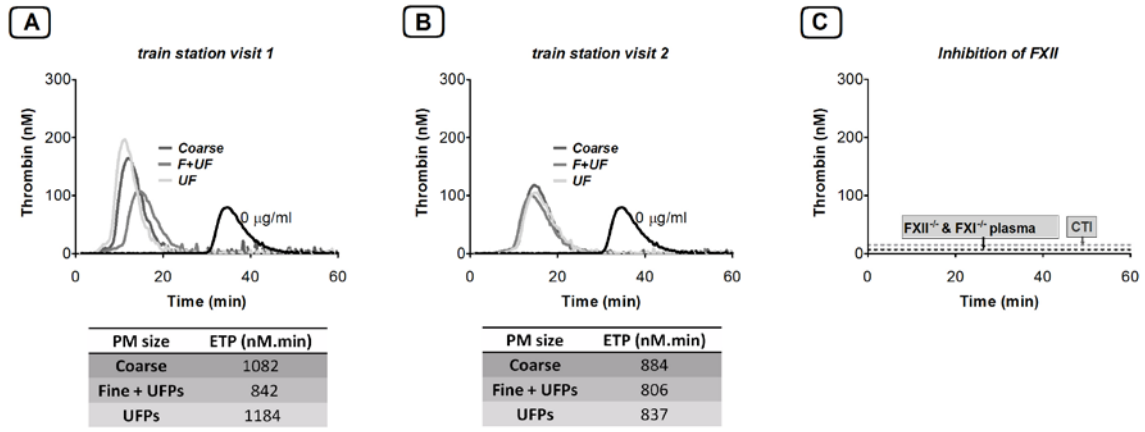
PM (µg/ml)	ETP (nM.min)
3.6	1230
1.8	1140
0.9	1019
0.45	870

PM (µg/ml)	ETP (nM.min)
3.6	1215
1.8	1095
0.9	956
0.45	840

Figure 4.1. ***In vitro plasma thrombin generation by coarse and fine PM***
Representative dose dependent thrombin generation curves and lag times, ETPs and Peak heights in human normal pooled platelet-poor plasma upon addition of PM. All measurements established in the presence of 4 µM phospholipids and ASIS (30 nM). Both coarse (n=4) and fine (n=4) PMs dose dependently increased thrombin generation in normal plasma (panels A and B). After inhibition of FXII either by adding of CTI to normal plasma or using $FXII^{-/-}$ or $FXI^{-/-}$ plasma, no thrombin generation was observed upon addition of PM (panel C). These results indicate that *in vitro* thrombin generation in plasma is enhanced by FXIIa formation in the presence of PM. Although coarse PMs had a slightly shorter lag times and increased ETPs and peak heights than fine PMs, the differences between those were not significant. Panel D: Lag time, Panel E: ETP, Panel F: peak height and normal plasma was presented as dashed lines. The data are presented as mean ± SD.

Note: Thrombin generation curves established from the average of triplicate measurements.

Results for the highest concentration of 3.6 µg/mL compared to normal plasma are shown in Fig. 4. 1 (panels D, E, and F). For each individual sample, the coarse fraction had a shorter lag time and higher ETP and peak height compared to the corresponding fine fraction. Thrombin generation results at the highest concentration of 3.6 µg/mL for PM biosamples of different size (coarse, fine+UFPs and UFPs) from one location (an underground train station) are depicted in Fig. 4. 2 (panels A and B). The UFPs fraction of PM biosamples from the first visit at the train station yielded the strongest thrombin generation in normal plasma, whereas for the second visit the response to coarse fraction was the strongest.



PM size	ETP (nM.min)
Coarse	1082
Fine + UFPs	842
UFPs	1184

PM size	ETP (nM.min)
Coarse	884
Fine + UFPs	806
UFPs	837

Figure 4.2. ***In vitro plasma thrombin generation by PM of different size***
Thrombin generation curves in human normal pooled platelet-poor plasma after addition of three different sizes of PM (collected at underground train station on 2 different time points; visit 1 & 2). Curves established in the presence of 4 µM phospholipids and ASIS (30 nM) for coarse, fine + UFPs and UFPs at concentration of 3.6 µg/mL. UFPs collected from train station at visit 1 yielded a strongest thrombin generation compared to Coarse and Fine + UFPs (panel A). Panel B: The size dependent thrombin generation indicated with respective thrombin curves of coarse, fine + UFPs and UFPs collected in same location at visit 2. These showed there is no size dependent thrombin generation in plasma upon addition of PM and it may indicate the changes in chemical composition. Consistently, inhibition of FXIIa in all conditions abolished thrombin generation (panel C).

Note: Thrombin generation curves established from the average of triplicate measurements.

The thrombin generation results in the presence of only phospholipids (4 µM; no ASIS) were comparable to the results derived from experiments with ASIS (*data not shown*). This suggests that the extrinsic pathway of coagulation in human plasma is not affected by PM. Furthermore, we analyzed whether FXII is activated in the presence of PM and determined thrombin formation in FXII and FXI-deficient human plasma. All applied PM completely failed to stimulate thrombin generation in the absence of FXII or FXI (Fig. 4.1C and Fig. 4.2C). Consistently, the specific FXIIa inhibitor (corn trypsin inhibitor; CTI: 40 µg/mL) abolished

thrombin generation in normal plasma (Fig. 4.1C and Fig. 4.2 C). Additionally, in the absence of phospholipids, no thrombin generation was observed in any experiment (*data not shown*).

The formation of FXIIa is increased in the presence of PM

In order to demonstrate the activation of FXII in the presence of PM, purified FXII was incubated with coarse PM at 4 different concentrations or buffer, which served as a negative control. After incubation, the generation of FXIIa was assessed using a chromogenic assay. In this purified system (only FXII protein and coarse PM), coarse PM clearly accelerated FXIIa formation in a dose dependent manner (Fig. 4.3A). In the presence of PK and HMWK, formation of FXIIa was increased in each corresponding dose of coarse PM (Fig. 4.3B).

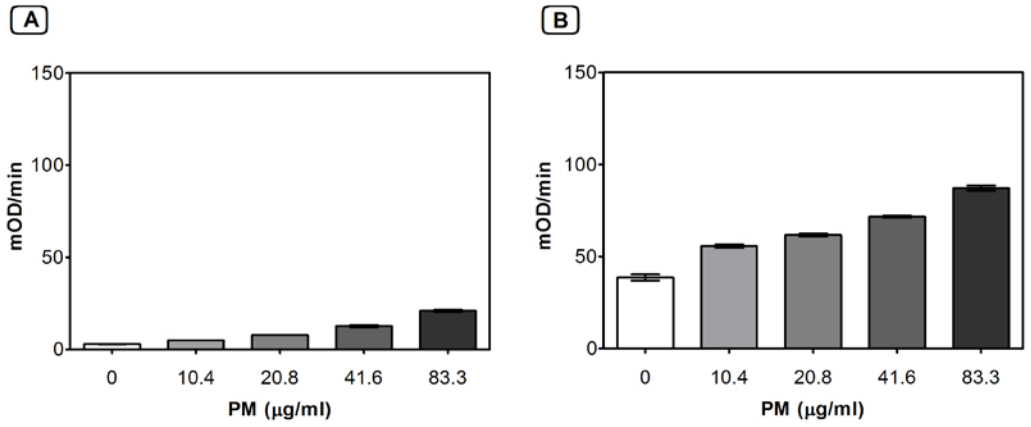


Figure 4.3. ***PM accelerates the formation of FXIIa***
FXIIa activation in the presence of PM (n=1) in a purified system determined from the cleavage of FXIIa substrate in the absence (panel A) and the presence (panel B) of PK and HMWK. Coarse PM increased the activity of FXIIa at all concentrations (panel A; p<0.0001) and in the presence of PK and HMWK, activation was increased for all corresponding concentrations of coarse PM (panel B; p<0.0001). The data are presented as mean ± SD.

UFPs Induce Early TF Pathway-mediated Procoagulant Shift upon Intratracheal Instillation in Mice

Given the distinct size of PM, UFPs are theoretically more likely to translocate into the blood stream than larger-sized PM fractions. Hereby, we undertook animal experiments with UFPs, collected near a Dutch roadside tunnel (3.6 µg/mL), which were intratracheally applied to WT (vehicle n=7; UFPs n=7) and $FXII^{-/-}$ mice (UFPs; n=8). Plasma was collected at 4 h post instillation in order to determine the early effects of UFPs on coagulation in mice.

In the presence of phospholipids (4 µM) and CTI (40 µg/ml), both WT (UFPs WT; Lag Time: 9.5 ± 4.6 min; ETP 185 ± 118 nM.min, Peak Height 7 ± 4 nM) and FXII$^{-/-}$ (UFPs FXII-/-; Lag Time: 8.4 ± 4.5 min; ETP 146 ± 125 nM.min, Peak Height 6 ± 5 nM) mice showed comparable levels of thrombin formed (Fig. 4.4: panels A, B, and C). Consistently, saline instillation in WT mice also showed limited thrombin generation (Vehicle WT; Lag Time: 28.6 ± 21.0 min; ETP 113 ± 72 nM.min, Peak Height 3 ± 2 nM).

This limited procoagulant effect was entirely inhibited upon the addition of ASIS (30 nM) and ASIS combined with CTI (suggesting no FXIa) [36] (*data not shown*), thus supporting the assumption of TF-mediated thrombin generation at 4 h post instillation. There was no thrombin generation observed in WT and FXII-/- mice at 20 h upon instillation of UFPs or saline in WT mice (*data not shown*).

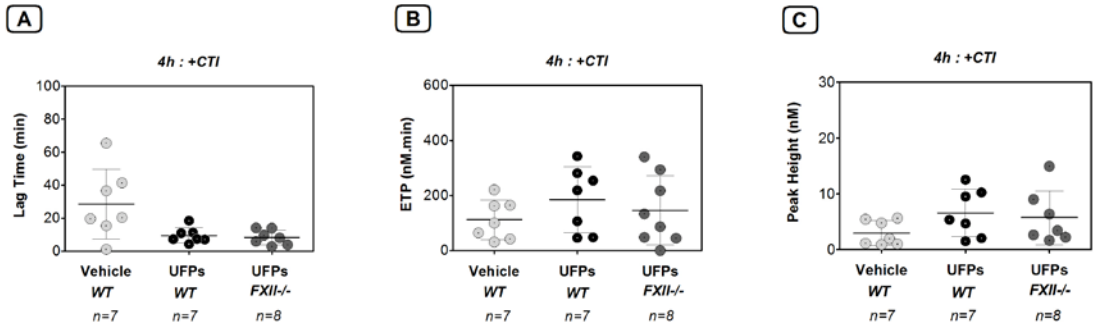


Figure 4.4. ***The effect of intratracheal administration UFPs on TF mediated thrombin generation in mouse plasma at 4 h post instillation***
Thrombin generation parameters in FXII$^{-/-}$ and WT mice plasma at 4 h after intratracheal instillation of UFPs, established in the presence of 4 µM phospholipids and CTI. The results demonstrate comparable, statistically non-significant, lag times, thrombin generation (ETP) and peak heights in FXII$^{-/-}$ (n=8) and WT mice (n=7) at 4 h post instillation of UFPs and saline (Vehicle) instillation in WT mice (panel A, B, C). The data are presented as mean ± SD.

Enhanced FXII activation is Critical for the Late Procoagulant Effects of UFPs in Mice

To better appreciate the procoagulant potential of PM in time, we also tested WT (n=8) and FXII$^{-/-}$ mice (n=8) at 20 h after intratracheal instillation of UFPs. Inhibition of the extrinsic coagulation route by ASIS (30nM) revealed an intrinsic pathway-dependent thrombin generation in WT mouse plasma (Lag Time: 9.9 ± 1.4 min, ETP: 285 ± 147 nM.min; Peak Height: 13 ± 8 nM; Fig. 4.5: panels D, E, and F) compared to vehicle (no thrombin generation; Fig. 4.5: panels D, E, and F). In contrast, no thrombin generation was observed in plasma from FXII$^{-/-}$ animals challenged with UFPs (Fig. 4.5A), suggesting a factor XII-dependency

Consistently, addition of CTI or CTI combined with ASIS to plasma of WT mice resulted in a complete diminution of thrombin generation (Fig. 4.5C), thus consolidating the evidence that the formation of FXIIa is enhanced in the presence of UFPs at a late time point (20h).

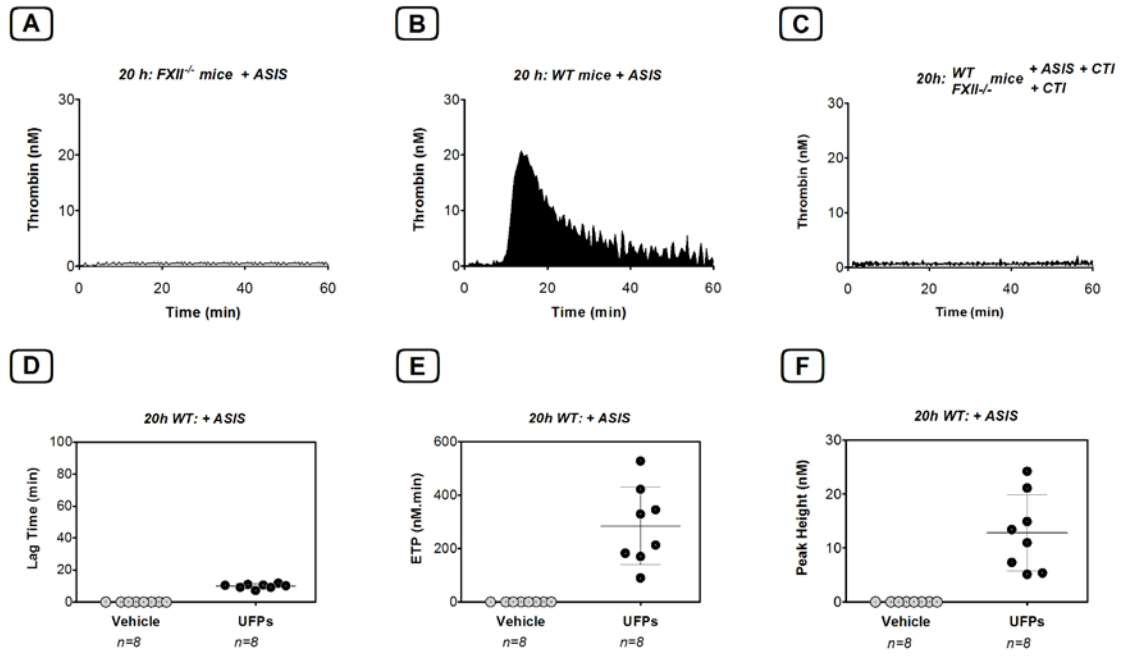


Figure 4.5. ***UFPs induces late thrombin formation, predominantly mediated via FXII***
Thrombin generation parameters in $FXII^{-/-}$ and WT mice plasma at 20 h after intratracheal administration of UFPs (n=8) and saline (Vehicle; n=8) instillation in WT mice, established in various conditions. Panel A: A representative thrombin generation curve of $FXII^{-/-}$ mouse plasma, measured in the addition of ASIS, which demonstrates that upon inhibition of the extrinsic route of coagulation no thrombin is formed. Panel B: A representative thrombin generation curve of WT mouse plasma, measured in the addition of ASIS, showing intense intrinsic pathway-dependent thrombin formation at 20 h post instillation of UFPs. Panel C: The involvement of FXII-driven thrombin generation in WT mice at 20 h is further supported by indicating that inhibition of the intrinsic pathway by the means of CTI and CTI combined ASIS (no FXIa) leads to a complete diminution of the curve. Panels D, E, and F: Shortened lag times, increased ETP and peak heights in WT mice compared to saline control (Vehicle) at 20 h after the instillation, implicating a role for increased FXII activation in mediating the delayed procoagulant effects of UFPs. The data are presented as mean ± SD.

Note: Lag times depicted as zero represent an absence of a thrombin generation curve. No statistic can be performed due to zero values in vehicle group.

Discussion

Both venous and arterial thrombotic events have been associated with exposure to PM [3, 5, 37, 38]. Experimental studies in animals, as well as in humans, have shown that thrombosis may be triggered by PM, affecting the blood coagulation system in various ways, including the induction of TF and increased platelet reactivity [10, 39-43].

The present study demonstrates the effects of PM on blood coagulation both *in vitro and* in mice. This report is the first to investigate the role of the contact activation pathway in mediating PM procoagulant actions. We provide new data demonstrating that UFPs exert a transient TF-dependent thrombin formation, responsible to induce an early procoagulant response upon challenge with PM, whereas enhanced FXII activation is essential to sustain this pro-thrombotic effect in mice. These findings are further consolidated by showing that PM accelerates the formation of FXIIa in human plasma. Moreover, in a purified system enhanced FXII activity was shown in the presence of PM in a dose dependent manner and the activity was increased in the presence of PK and HMWK.

Although our data do not provide direct evidence, it may be postulated that PM acts as a surface for coagulation reactions, including assembly of contact proteins. The contribution of PM in this reaction may in part depend on its chemical composition. It was indeed demonstrable that PM collected from the same location at two different time points did not show consistent thrombin generation results in human plasma. Although the chemical analysis of PM in relation to procoagulant activity was beyond the scope of this study, there is published data to document various effects. PM consists of transition metals such as Nickel (Ni) and Ferro (Fe), sulfates, nitrates, and black carbon [44, 45]. Sangani et al. recently showed that a whole blood coagulation time decreased after addition of PM to human blood compared to addition of buffer solution alone, an effect related to the presence of metal sulfates [46].

In the upper airways, local reactions in the lung, involving pulmonary inflammation, TF production and oxidative stress on macrophages and lung epithelial cells [8, 12, 47, 48], may underlie activation of the TF-mediated blood coagulation by PM. Although the mucocilary clearance will remove all particles larger than 6 μm, it is suggested that the efficiency of this system is very low for UFPs and at the deeper lung regions, the clearance of particles will rely in part on phagocytic uptake [49]. In contrast to larger particles, the macrophage uptake of UFPs seem to be less effective [50]. Using different animal species, it has been demonstrated that part of UFPs transmigrates from the lungs into the blood circulation [13-19], although the

efficiency of translocation may be low, around 1- 2.5% reaches the circulating blood in human and rodents [21, 49, 51]. However, it should be noted that translocation remains controversial in humans since other investigators have not confirmed this mechanism to be operational [20, 52], probably depending on differences in techniques. In the present study, the main focus was on the question whether UFPs activate coagulation, particularly, intrinsic pathway of coagulation when they enter the bloodstream, assuming that translocation would occur. To test this hypothesis, we applied the intratracheal instillation method which is a validated [53] and commonly used procedure in PM related studies [9, 54-56], although one should keep in mind that this is different from exposure to environmental pollutants. In selecting the dose ranges for PM application, we followed the reasoning in the paper by Stoeger and colleagues, demonstrating that 3-5 μg UFPs would be a relevant concentration to apply in mice, extrapolating urban environmental concentrations in man to the surface area exposed in mice [57].

Assuming translocation, another issue is the retention of UFPs in the circulation. In a very recent study Hirn et al [58] addressed the fate of gold labeled nanoparticles (GNPs) of different size and surface charge after intravenous administration in rats. They still found small but detectable amounts of the administered GNPs of all sizes in the blood at 24 h after administration. Their data suggested retention of GNPs in blood and their localization on cells or in serum to be size dependent. Similar mechanisms may explain the persistent presence of PM related activity in blood after intratracheal exposure, probably associated with phospholipid surfaces (cells, microparticles) to increase the formation of FXIIa over time. The current data are consistent with results from experimental exposures in healthy human subjects, where diesel engine exhaust exposure significantly increased the *ex vivo* thrombus generation in a Badimon chamber as compared to filtered air [10]. In this system peripheral venous blood from the subjects is directly applied to strips of endothelial denuded pig aorta at high and low flow rates, mimicking plaque rupture in large and small arteries. The increase in thrombus following inhalation of diesel engine exhaust particles was related to increased platelet-leukocyte adhesion, but this was not entirely explained by this effect [10]. We suggest that the presently identified enhanced activation of FXII in the presence of UFPs is the major additional factor behind the enhanced tendency to thrombus formation in the Badimon chamber *ex vivo* model. In conclusion, we have shown that PM enhances the formation of FXIIa *in vitro* and that traffic-related UFPs induce a slow but significant increment of thrombin generation *in vivo*, which is dependent on contact activation. While previous studies demonstrated early activation of coagulation to be TF mediated, our data extend the

significance of FXII driven coagulation activity for sustaining an increased level of thrombin generation in time. Given the potential of thrombin to act in a pro-atherogenic manner against a background of inflammation, these mechanisms may be relevant for the increased risk of thrombosis as well as atherosclerosis in humans, exposed to PM in daily life [59].

Addendum

E. Kilinç, H.M.H. Spronk, T. Renné, and H. ten Cate designed the study, and drafted the manuscript and R. van Oerle, C. Oschatz co-ordinated and performed laboratory investigations. J. I Borissof contributed to drafting the manuscript. M. E. Gerlofs-Nijland, N.A. Janssen, F. R. Cassee supplied the PM samples, participated in the study design, data interpretation and drafting of the manuscript. T. Sandström has been the co-ordinator of the European HEPMEAP project that supplied the PM samples collected on PUF. All authors contributed to the writing and critically reviewed the manuscript.

Acknowledgements

This study was financially supported by the Netherlands Heart Foundation (grant number: 2006B064) and VR grant K2010-64X21462013 to T. Renné. Marie Curie fellowship from the European Commission was granted to Dr. Borissoff (MEST-CT-2005-020706). We would like to thank Diana Fens and Patricia Pluijmen for laboratory assistance and logistic support. PM samples collected on PUF were collected in the framework of a grant from the European Union under the 5th Framework Programme, Quality of Life and Management of Living Resources, Key Action 4: Environment and Health by the HEPMEAP project contract QLRT-1999-01582 (www.hepmeap.org). Samples from the underground were collected within the framework of the RAPTES project, funded by the RIVM strategic research program.

References

1 Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*. 2004; **109**: 2655-71.

2 Harrison RM, Yin J. Particulate matter in the atmosphere: which particle properties are important for its effects on health? *Sci Total Environ*. 2000; **249**: 85-101.

3 Dales RE, Cakmak S, Vidal CB. Air pollution and hospitalization for venous thromboembolic disease in Chile. *J Thromb Haemost*. 2010; **8**: 669-74.

4 Hoek G, Brunekreef B, Fischer P, van Wijnen J. The association between air pollution and heart failure, arrhythmia, embolism, thrombosis, and other cardiovascular causes of death in a time series study. *Epidemiology*. 2001; **12**: 355-7.

5 Baccarelli A, Martinelli I, Zanobetti A, Grillo P, Hou LF, Bertazzi PA, Mannucci PM, Schwartz J. Exposure to particulate air pollution and risk of deep vein thrombosis. *Arch Intern Med*. 2008; **168**: 920-7.

6 Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, Sandstrom T, Blomberg A, Newby DE. Adverse cardiovascular effects of air pollution. *Nat Clin Pract Cardiovasc Med*. 2009; **6**: 36-44.

7 Lippi G, Favaloro EJ, Franchini M, Guidi GC. Air pollution and coagulation testing: a new source of biological variability? *Thromb Res*. 2008; **123**: 50-4.

8 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.

9 Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest*. 2007; **117**: 2952-61.

10 Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. Diesel exhaust inhalation increases thrombus formation in man. *Eur Heart J*. 2008; **29**: 3043-51.

11 Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandstrom T, Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation*. 2005; **112**: 3930-6.

12 Tornqvist H, Mills NL, Gonzalez M, Miller MR, Robinson SD, Megson IL, Macnee W, Donaldson K, Soderberg S, Newby DE, Sandstrom T, Blomberg A. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *Am J Respir Crit Care Med*. 2007; **176**: 395-400.

13 Chen J, Tan M, Nemmar A, Song W, Dong M, Zhang G, Li Y. Quantification of extrapulmonary translocation of intratracheal-instilled particles in vivo in rats: effect of lipopolysaccharide. *Toxicology*. 2006; **222**: 195-201.

14 Furuyama A, Kanno S, Kobayashi T, Hirano S. Extrapulmonary translocation of intratracheally instilled fine and ultrafine particles via direct and alveolar macrophage-associated routes. *Arch Toxicol*. 2009; **83**: 429-37.

15 Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A*. 2002; **65**: 1513-30.

16 Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med*. 2001; **164**: 1665-8.

17 Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A*. 2002; **65**: 1531-43.

18 Takenaka S, Karg E, Roth C, Schulz H, Ziesenis A, Heinzmann U, Schramel P, Heyder J. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environ Health Perspect*. 2001; **109 Suppl 4**: 547-51.

19 Kilinç E, Rudež G, Spronk HMH, Nemmar A, de Maat MPM, ten Cate H, Hoylaerts MF. Particles, Coagulation, and Thrombosis *Cardiovascular Effects of Inhaled Ultrafine and Nano-Sized Particles*. 2011: 405-21.

20 Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, Macnee W, Millar AM, Donaldson K, Newby DE. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *Am J Respir Crit Care Med*. 2006; **173**: 426-31.

21 Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation*. 2002; **105**: 411-4.

22 Zhuo R, Siedlecki CA, Vogler EA. Autoactivation of blood factor XII at hydrophilic and hydrophobic surfaces. *Biomaterials*. 2006; **27**: 4325-32.

23 Maas C, Govers-Riemslag JW, Bouma B, Schiks B, Hazenberg BP, Lokhorst HM, Hammarstrom P, ten Cate H, de Groot PG, Bouma BN, Gebbink MF. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. *J Clin Invest*. 2008; **118**: 3208-18.

24 Muller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renne T. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*. 2009; **139**: 1143-56.

25 van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslag JW, Cosemans JM, Kuijpers MJ, Spronk HM, Watson SP, Renne T, Heemskerk JW. Dual role of collagen in factor XII-dependent thrombus formation. *Blood*. 2009; **114**: 881-90.

26 Gailani D, Renne T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *J Thromb Haemost*. 2007; **5**: 1106-12.

27 Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med*. 2005; **202**: 271-81.

28 Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, Renne C, Gailani D, Nieswandt B, Renne T. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med*. 2006; **203**: 513-8.

29 Doggen CJ, Rosendaal FR, Meijers JC. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. *Blood*. 2006; **108**: 4045-51.

30 Endler G, Marsik C, Jilma B, Schickbauer T, Quehenberger P, Mannhalter C. Evidence of a U-shaped association between factor XII activity and overall survival. *J Thromb Haemost*. 2007; **5**: 1143-8.

31 Govers-Riemslag JW, Smid M, Cooper JA, Bauer KA, Rosenberg RD, Hack CE, Hamulyak K, Spronk HM, Miller GJ, ten Cate H. The plasma kallikrein-kinin system and risk of cardiovascular disease in men. *J Thromb Haemost*. 2007; **5**: 1896-903.

32 Gerlofs-Nijland ME, Dormans JA, Bloemen HJ, Leseman DL, John A, Boere F, Kelly FJ, Mudway IS, Jimenez AA, Donaldson K, Guastadisegni C, Janssen NA, Brunekreef B, Sandstrom T, van Bree L, Cassee FR. Toxicity of coarse and fine particulate matter from sites with contrasting traffic profiles. *Inhal Toxicol*. 2007; **19**: 1055-69.

33 Kim S, Jaques PA, Chang MC, Froines JR, Sioutas C. Versatile aerosol concentration enrichment system (VACES) for simultaneous in vivo and in vitro evaluation of toxic effects of ultrafine, fine and coarse ambient particles - Part I: Development and laboratory characterization. *Journal of Aerosol Science*. 2001; **32**: 1281-97.

34 Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, Lecompte T, Beguin S. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb*. 2002; **32**: 249-53.

35 Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost*. 2008; **6**: 125-31.

36 Loubele ST, Spek CA, Leenders P, van Oerle R, Aberson HL, van der Voort D, Hamulyak K, Petersen LC, Spronk HM, ten Cate H. Active site inhibited factor VIIa attenuates myocardial ischemia/reperfusion injury in mice. *J Thromb Haemost*. 2009; **7**: 290-8.

37 Baccarelli A, Martinelli I, Pegoraro V, Melly S, Grillo P, Zanobetti A, Hou L, Bertazzi PA, Mannucci PM, Schwartz J. Living near major traffic roads and risk of deep vein thrombosis. *Circulation*. 2009; **119**: 3118-24.

38 Johnson D, Parker JD. Air pollution exposure and self-reported cardiovascular disease. *Environ Res*. 2009; **109**: 582-9.

39 Khandoga A, Stoeger T, Khandoga AG, Bihari P, Karg E, Ettehadieh D, Lakatos S, Fent J, Schulz H, Krombach F. Platelet adhesion and fibrinogen deposition in murine microvessels upon inhalation of nanosized carbon particles. *J Thromb Haemost*. 2010; **8**: 1632-40.

40 Kooter IM, Frederix K, Spronk HM, Boere AJ, Leseman DL, van Steeg H, ten Cate H, Cassee FR. Lung inflammation and thrombogenic responses in a time course study of Csb mice exposed to ozone. *J Appl Toxicol*. 2008; **28**: 779-87.

41 Nemmar A, Hoet PH, Dinsdale D, Vermylen J, Hoylaerts MF, Nemery B. Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. *Circulation*. 2003; **107**: 1202-8.

42 Nemmar A, Hoylaerts MF, Hoet PH, Dinsdale D, Smith T, Xu H, Vermylen J, Nemery B. Ultrafine particles affect experimental thrombosis in an in vivo hamster model. *Am J Respir Crit Care Med*. 2002; **166**: 998-1004.

43 Rudez G, Janssen NA, Kilinc E, Leebeek FW, Gerlofs-Nijland ME, Spronk HM, ten Cate H, Cassee FR, de Maat MP. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect*. 2009; **117**: 995-1001.

44 Ripanucci G, Grana M, Vicentini L, Magrini A, Bergamaschi A. Dust in the underground railway tunnels of an Italian town. *J Occup Environ Hyg*. 2006; **3**: 16-25.

45 Seaton A, Cherrie J, Dennekamp M, Donaldson K, Hurley JF, Tran CL. The London Underground: dust and hazards to health. *Occup Environ Med*. 2005; **62**: 355-62.

46 Sangani RG, Soukup JM, Ghio AJ. Metals in air pollution particles decrease whole-blood coagulation time. *Inhal Toxicol*. 2010; **22**: 621-6.
47 Brown DM, Hutchison L, Donaldson K, Stone V. The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *Am J Physiol Lung Cell Mol Physiol*. 2007; **292**: L1444-51.
48 Gilmour PS, Morrison ER, Vickers MA, Ford I, Ludlam CA, Greaves M, Donaldson K, MacNee W. The procoagulant potential of environmental particles (PM10). *Occup Environ Med*. 2005; **62**: 164-71.
49 Schmid O, Moller W, Semmler-Behnke M, Ferron GA, Karg E, Lipka J, Schulz H, Kreyling WG, Stoeger T. Dosimetry and toxicology of inhaled ultrafine particles. *Biomarkers*. 2009; **14 Suppl 1**: 67-73.
50 Tabata Y, Ikada Y. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage. *Biomaterials*. 1988; **9**: 356-62.
51 Moller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, Haussinger K, Kreyling WG. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. *Am J Respir Crit Care Med*. 2008; **177**: 426-32.
52 Brown JS, Zeman KL, Bennett WD. Regional deposition of coarse particles and ventilation distribution in healthy subjects and patients with cystic fibrosis. *J Aerosol Med*. 2001; **14**: 443-54.
53 Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdorster G, Salem H, Schlesinger RB. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci*. 2000; **55**: 24-35.
54 Cozzi E, Wingard CJ, Cascio WE, Devlin RB, Miles JJ, Bofferding AR, Lust RM, Van Scott MR, Henriksen RA. Effect of ambient particulate matter exposure on hemostasis. *Transl Res*. 2007; **149**: 324-32.
55 Emmerechts J, Alfaro-Moreno E, Vanaudenaerde BM, Nemery B, Hoylaerts MF. Short-term exposure to particulate matter induces arterial but not venous thrombosis in healthy mice. *J Thromb Haemost*. 2010; **8**: 2651-61.
56 Nemmar A, Hoet PH, Vandervoort P, Dinsdale D, Nemery B, Hoylaerts MF. Enhanced peripheral thrombogenicity after lung inflammation is mediated by platelet-leukocyte activation: role of P-selectin. *J Thromb Haemost*. 2007; **5**: 1217-26.
57 Stoeger T, Reinhard C, Takenaka S, Schroeppel A, Karg E, Ritter B, Heyder J, Schulz H. Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. *Environ Health Perspect*. 2006; **114**: 328-33.
58 Hirn S, Semmler-Behnke M, Schleh C, Wenk A, Lipka J, Schaffler M, Takenaka S, Moller W, Schmid G, Simon U, Kreyling WG. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. *Eur J Pharm Biopharm*. 2011; **77**: 407-16.
59 Borissoff JI, Spronk HM, Heeneman S, ten Cate H. Is thrombin a key player in the 'coagulation-atherogenesis' maze? *Cardiovasc Res*. 2009; **82**: 392-403.

CHAPTER 5

EXPOSURE TO COMBUSTION DERIVED NANOPARTICLES DOES NOT ALTER TISSUE FACTOR MEDIATED THROMBIN GENERATION IN HEALTHY YOUNG ADULTS

Kilinç, E., Spronk, H.M.H., Serroyen, J., Cassee, F.R., Mills, N.L., ten Cate, H.

Submitted

Abstract

Diesel exhaust is a major contributor to urban particulate matter and may be an important trigger for thrombotic cardiovascular events such as myocardial infarction, but the individual pollutants responsible for this effect have not been established. We hypothesised that the prothrombotic effect of diesel exhaust are due to an effect of combustion-derived nanoparticles on tissue factor (TF)-mediated blood coagulation. Sixteen healthy volunteers were exposed to (i) dilute diesel exhaust, (ii) pure carbon nanoparticulate, (iii) filtered diesel exhaust, or (iv) filtered air, in a randomised double blind cross-over study. Following each exposure at 2, 6 and 24 hours, blood samples were collected and plasma thrombin generation assesed in the presence or absence of TF. Compared to filtered air, inhalation of diesel exhaust, carbon nanoparticulate or filtered exhaust had no effect on TF-mediated thrombin generation at any time point. Our findings suggest that the prothrombotic effects of diesel exhaust are not mediated through the TF-driven extrinsic pathway of coagulation in healthy persons. Ultrafine particles (<0.1 μm) may exert direct effects on the instrinsic pathway of coagulation to explain the association between particulate matter and thrombotic events.

Introduction

Exposure to particulate matter (PM) has been linked to increased thrombotic and adverse cardiovascular events [1-3]. Diesel engine exhaust (DEE) is a major contributor to urban air pollution with particulate matter, gaseous pollutants and volatiles all potentially harmful to human health. We have previously demonstrated that exposure to DEE impairs vascular function [4], and is pro-thrombotic in both healthy volunteers [5] and patients with coronary heart disease [6]. Experimental and clinical research studies suggest a role for gaseous and particle air pollutants in mediating the cardiovascular effects of air pollution [7, 8]. Fine (0.15 - 2.5 μm aerodynamic diameter) and ultrafine particles (<0.1 μm; UFPs) are thought to be the most toxic component of DEE [9]. UFPs may be more hazardous than fine PM because of their small size and greater surface area, and it remains plausible that these particles may translocate into in the blood circulation [10-12].

We hypothesised that the prothrombotic effect of DEE are due to an effect of combustion-derived nanoparticles on tissue factor (TF)-mediated blood coagulation. We assessed the effect of exposure to combustion-derived nanoparticles with and without gaseous co-pollutants on thrombin generation in plasma in man.

Material and Methods

Study design and Exposure

Using a specially designed human exposure chamber and particle filtration system [13], we compared the effects of dilute diesel exhaust with the gaseous components alone, and with 'clean' carbon nanoparticles. Sixteen healthy, nonsmoking males (18 to 32 years of age) participated in the study and subjects attended on 4 separate occasions at least 2 weeks apart. Using a double-blind randomized cross-over design subjects were exposued to filtered air, carbon nanoparticles (70 μg/m^3), diesel exhaust (350 μg/m^3) and filtered diesel exhaust from which the particulate phase was removed. On each occasion, each subject was exposed for 2 hours while they performed moderate exercise (minute ventilation 25 L/min/m^2) on a bicycle ergometer with rest at 15-min intervals. The study was performed with approval from the local research ethics committee (University of Edinburgh), in accordance with the Declaration of Helsinki, and with written informed consent of all volunteers.

Blood Collection

Venous blood was collected into citrate before (t=0), and 2, 6 and 24 hours after each exposure. Samples were kept on ice before being centrifuged at 2000 *g* for 30 min at 4°C and platelet-poor plasma was stored at -80°C before analysis.

Thrombin generation

Plasma thrombin generation was measured by means of the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, the Netherlands) [14] and measurements were performed in the presence of 4 μM phospholipids and 1 pM TF (ppp low reagent; Thrombinoscope BV) according to the following protocol: 20 μl of reagent, 80 μl of plasma incubated for 10 min at 37 °C. The reaction was started upon addition of calcium and fluorogenic substrate (20 μl) and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV) as described [15]. Three parameters were derived from the thrombin generation curves: lag time, peak height and endogenous thrombin potential (ETP, area under the curve). Normal pool plasma (prepared by two centrifugation steps: the first at 2,000x g for 15 minutes and the second at 11,000x g for 10 min) was analyzed on each plate. Thrombin generation parameters were normalized and given as ratio to normal pool plasma.

Statistical analysis

Data were analyzed using a linear mixed model, which included a random intercept, in order to take the correlations due to repeated measurements on the same subject into account. All statistical analyses were performed using SPSS software version 18 (SPSS Inc. Chicago, Ill., USA). P-values less than 0.05 were considered as statistically significant.

Results

In healthy volunteers exposure to DEE at concentrations encountered in rush-hour traffic, did not significantly alter TF-mediated thrombin generation with lag times, ETP and peak height unchanged at any time point (Table 5.1). Consistent with this finding, filtered DEE (gaseous pollutants only) and carbon nanoparticles did not significantly affect TF-mediated coagulation compared to filtered air.

Table 5.1. Thrombin generation parameters after exposure to filtered air, diesel exhaust, filtered diesel exhaust and carbon nanoparticles

	Filtered Air				Diesel Exhaust				Filtered Diesel Exhaust				Carbon			
Time, hours	0	2	6	24	0	2	6	24	0	2	6	24	0	2	6	24
Lag time (min)	6.8±1.5	7.1±1.2	7.5±1.7	7.0±1.2	7.0±1.5	7.4±2.2	7.8±2.2	7.5±2.0	7.2±1.4	7.1±1.4	7.5±1.1	7.4±1.6	7.9±1.7	7.7±1.3	8.1±1.4	8.1±1.4
ETP (%)	78±19	77±21	79±18	80±27	78±19	77±21	79±18	80±27	75±19	79±23	82±22	75±24	73±18	82±23	82±23	73±14
Peak Height (%)	73±29	77±29	73±19	68±19	86±31	73±21	74±26	71±25	68±24	78±30	78±26	74±37	65±20	86±31	78±28	67±20

Values are reported as mean ± SD (n=16)

Discussion

In several studies, we have attempted to identify the components responsible for the adverse thrombotic cardiovascular effects of air pollution. In contrast to our earlier experimental study in which we demonstrated an early activation of TF-mediated blood coagulation in mice following exposure to DEE [16], the present study did not suggest any effect of the particulate or gaseous components in DEE on the extrinsic blood coagulation in humans. Given thrombin generation ultimately yields fibrin formation, our data is in agreement with a parallel study in which DEE exposure did not alter fibrin structure in healthy humans [17].

The particle concentrations of 300 to 350 μg/m^3 delivered in our experimental chamber in the present study are similar to those encountered during rush-hour traffic in large cities [18], however, the mass and gaseous pollutant concentrations used in our previous animal studies were more than 6-fold higher at 1900 μg/m^3 [16]. It has previously been demonstrated that DEE particles when added directly to rat blood shortened PT and aPTT in a dose dependent manner [19]. It is possible that any effect of exposure DEE in humans at realistic concentrations are counteracted by the natural anticoagulant forces, including thrombin mediated protein C activation [20].

Our previous *in vitro* studies suggest particles of different sizes can increase thrombin generation in human plasma via the formation of activated factor XII (FXII). This effect was replicated *in vivo* where the instillation of low dose UFPs (3.6 μg/ml in saline) increased FXII-mediated thrombin generation in mice compared to saline placebo [21]. Earlier studies with FXII deficient mice suggest a role for FXII in formation of arterial thrombosis [22]. In other observations from our present study exposure to DEE significantly increased *ex vivo* arterial thrombus formation in a Badimon chamber compared to filtered air [5]. Emmerechts et al. recently found a preferential effect of intra-tracheal PM on arterial rather than venous thrombosis in mice [23]. These studies taken together suggest that FXII may mediate the effects of urban PM on arterial thrombosis and further studies are necessary to assess this hypothesis in man.

In conclusion, exposure to DEE, filtered DEE, or carbon nanoparticles does not alter TF-mediated thrombin generation in man. These findings do not rule out a prothrombotic effect of DEE under different conditions, where FXII driven coagulation would prevail, such as after exposure to platelet polyphosphates or in contact with artificial surfaces. There is strong evidence from our earlier studies that FXII driven coagulation pathways may mediate the

effect of particles on thrombin generation in time and may explain the increase in thrombotic cardiovascular disease associated with environmental particles.

Acknowledgements

This study was supported by the Netherlands Heart Foundation (grant number: 2006B064) and a British Heart Foundation Programme Grant (PG/05/003). Dr Mills is supported by an Intermediate Clinical Research Fellowship from the British Heart Foundation (FS/10/024). We thank Pamela Dawson, Daan Leseman, Finny Paterson and all the staff at the Wellcome Trust Clinical Research Facility, Edinburgh, for their assistance with the studies.

References

1 Kwon HJ, Cho SH, Nyberg F, Pershagen G. Effects of ambient air pollution on daily mortality in a cohort of patients with congestive heart failure. *Epidemiology*. 2001; **12**: 413-9.

2 Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. *Circulation*. 2001; **103**: 2810-5.

3 Pope CA, 3rd, Muhlestein JB, May HT, Renlund DG, Anderson JL, Horne BD. Ischemic heart disease events triggered by short-term exposure to fine particulate air pollution. *Circulation*. 2006; **114**: 2443-8.

4 Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandstrom T, Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation*. 2005; **112**: 3930-6.

5 Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. Diesel exhaust inhalation increases thrombus formation in man. *Eur Heart J*. 2008; **29**: 3043-51.

6 Mills NL, Tornqvist H, Gonzalez MC, Vink E, Robinson SD, Soderberg S, Boon NA, Donaldson K, Sandstrom T, Blomberg A, Newby DE. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N Engl J Med*. 2007; **357**: 1075-82.

7 Kilinc E, Schulz H, Kuiper GJ, Spronk HM, Ten Cate H, Upadhyay S, Ganguly K, Stoeger T, Semmler-Bhenke M, Takenaka S, Kreyling WG, Pitz M, Reitmeir P, Peters A, Eickelberg O, Wichmann HE. The procoagulant effects of fine particulate matter in vivo. *Particle and fibre toxicology*. 2011; **8**: 12; author reply.

8 Rudez G, Janssen NA, Kilinc E, Leebeek FW, Gerlofs-Nijland ME, Spronk HM, ten Cate H, Cassee FR, de Maat MP. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect*. 2009; **117**: 995-1001.

9 Sydbom A, Blomberg A, Parnia S, Stenfors N, Sandstrom T, Dahlen SE. Health effects of diesel exhaust emissions. *Eur Respir J*. 2001; **17**: 733-46.

10 Furuyama A, Kanno S, Kobayashi T, Hirano S. Extrapulmonary translocation of intratracheally instilled fine and ultrafine particles via direct and alveolar macrophage-associated routes. *Arch Toxicol*. 2009; **83**: 429-37.

11 Kilinç E, Rudež G, Spronk HMH, Nemmar A, de Maat MPM, ten Cate H, Hoylaerts MF. Particles, Coagulation, and Thrombosis In: Cassee FR, Mills NL, Newby DE, eds. *Cardiovascular Effects of Inhaled Ultrafine and Nano-Sized Particles*. Chicester: John Wiley and Sons Ltd, 2011.

12 Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med*. 2001; **164**: 1665-8.

13 Mills NL, Miller MR, Lucking AJ, Beveridge J, Flint L, Boere AJ, Fokkens PH, Boon NA, Sandstrom T, Blomberg A, Duffin R, Donaldson K, Hadoke PW, Cassee FR, Newby DE. Combustion-derived nanoparticulate induces the adverse vascular effects of diesel exhaust inhalation. *European heart journal*. 2011.

14 Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, Lecompte T, Beguin S. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb*. 2002; **32**: 249-53.

15 Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost*. 2008; **6**: 125-31.

16 Kooter IM, Gerlofs-Nijland ME, Boere AJ, Leseman DL, Fokkens PH, Spronk HM, Frederix K, Ten Cate H, Knaapen AM, Vreman HJ, Cassee FR. Diesel engine exhaust initiates a sequence of pulmonary and cardiovascular effects in rats. *J Toxicol*. 2010; **2010**: 206057.

17 Metassan S, Routledge MN, Lucking AJ, Uitte de Willige S, Philippou H, Mills NL, Newby DE, Ariens RA. Fibrin clot structure remains unaffected in young, healthy individuals after transient exposure to diesel exhaust. *Part Fibre Toxicol*. 2010; **7**: 17.

18 Health UNEPaW, Report. O. Air pollution in the world's megacities. United Nations Environment Programme and World Health Organization Report, 1994, 5-37.

19 Nemmar A, Al-Salam S, Zia S, Dhanasekaran S, Shudadevi M, Ali BH. Time-course effects of systemically administered diesel exhaust particles in rats. *Toxicol Lett*. 2010; **194**: 58-65.

20 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.

21 Kilinc E, van Oerle R, Borissoff JI, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HM. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *Journal of thrombosis and haemostasis : JTH*. 2011; **9**: 1359-67.

22 Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med*. 2005; **202**: 271-81.

23 Emmerechts J, Alfaro-Moreno E, Vanaudenaerde BM, Nemery B, Hoylaerts MF. Short-term exposure to particulate matter induces arterial but not venous thrombosis in healthy mice. *J Thromb Haemost*. 2010; **8**: 2651-61.

CHAPTER 6

EXPOSURE TO BIODIESEL EXHAUST TRIGGERS ATHEROSCLEROTIC PLAQUE DESTABILIZATION THROUGH ACCELERATED OXIDANT STRESS AND APOPTOSIS IN THE ARTERIAL VESSEL WALL

Kilinç, E*., Borissoff, J.I.*, Haegens, A., Kooter, I.M., Jedynska, A.D., Tjwa, D.J.T.H., Spronk, H.M.H., ten Cate, H.

Submitted

***shared first authorship**

Abstract

Long-term exposure to air pollutants has been unequivocally linked to increased risk of cardiovascular morbidity and mortality. Biodiesels are widely introduced as renewable fuels which do not contribute to the carbon dioxide burden and produce reduced engine emissions. While particulate matter (PM) derived from diesel exhaust have been documented to be pro-atherogenic in animal studies, the effects of biodiesel fuels remain unknown.

We hypothesize that exposure to biodiesel exhaust will result in diminished vascular inflammation, atherosclerosis progression and more stable atherosclerotic plaque phenotype (vs. diesel exhaust).

In a carotid cuff atherosclerosis model, we tested the effects of exposure to biodiesel PM (B30) vs. diesel PM (B0) vs. saline (control) on atherosclerosis plaque progression. We placed silastic collars around the common carotid arteries in LDLR $^{-/-}$ mice at week 2 in the course of an 8-week high-fat diet regimen. All mice were intratracheally instilled with saline, PM B0 or PM B30 once weekly. Exposure to both B0 and B30 did not result in exacerbation of atherosclerosis growth. However, exposure to B30 PM affected atherosclerotic plaque composition, inducing a vulnerable lesion phenotype with signs of fibrous cap thinning, enhanced plaque necrosis and decreased total collagen content. Furthermore, exposure to biodiesel triggered a substantial loss of VSMC in tunica media, which strongly correlated with the apoptosis index in the arterial vessel wall (Pearson $r=0.7$, $p<0.05$)

This study provides novel evidence suggesting that exposure to low concentrations of biodiesel exhaust dramatically modulates atherosclerotic plaque composition, resulting in unstable plaque phenotype through enhanced pro-oxidant and pro-apoptotic mechanisms.

Introduction

Air pollution is a major environmental health hazard. Besides the adverse effects, which ambient particles primarily exert on the respiratory system, there is a substantial body of evidence suggesting that exposure to fine and ultrafine particulate matter (PM) can also have a negative impact on cardiovascular health [1]. Atherosclerosis is widely recognized as a multifactorial progressive inflammatory vascular disease [2]. The exact underlying mechanisms through which PM contributes to atherosclerotic plaque formation and progression remain unclear to date, animal studies, however, have clearly documented its pro-atherogenic potential [3-5]. Numerous epidemiological studies showed consistent results, indicating a strong positive association between long-term exposure to PM air pollution and increased risk of cardiovascular morbidity [6-8] and mortality [9-12].

With development of renewable energy resources, the demand for alternative type of fuels, such as biodiesel, is rapidly growing worldwide. Biodiesel is derived through trans-esterification of vegetable oils and animal fats and is provided either in pure (B100) or blended form (a fuel mix with petroleum diesel). Biodiesel consumption is linked to significantly lower sulfur, carbon monoxide and gas emissions and ~50% reduction in particulate matter emissions in comparison to conventional petroleum diesel [13]. Despite biodiesel's superior emissions profile, there has been limited research carried out to study its effects on biological systems *in vivo*. Recent animal studies demonstrate that exposure to petroleum diesel exhaust promotes systemic inflammation, enhanced oxidative stress and vulnerable atherosclerotic plaque phenotype [14]. However, the effects of biodiesel exhaust on atherosclerosis plaque onset and progression have never been addressed before.

Hence, we hypothesized that in an animal model of atherosclerosis, exposure to biodiesel exhaust will result in diminished vascular inflammation, atherosclerosis progression and more stable atherosclerotic plaque phenotype when compared to conventional diesel exhaust.

Materials and methods

Animal model

Eight-week-old female LDL receptor knockout (LDLR $^{-/-}$) mice on C57BL/6J background were obtained from Charles River Laboratories (Maastricht, The Netherlands). All animal experiments were approved by the Animal Care and Use Committee of Maastricht University (Maastricht, The Netherlands) and were performed in compliance with the guidelines established by the Dutch Council on Animal Care.

Atherosclerosis model and exposure to PM

Atherosclerosis development was induced through placement of perivascular collars on the common carotid arteries as previously described [15]. All mice were provided with a high-cholesterol diet (15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 20% casein, free of cholate, total fat content 16%; Hope Farms, Woerden, The Netherlands) and water *ad libitum* throughout all experiments. Mice were randomly assigned to three groups (n=9 per group): 1. Exposure to saline (control group); 2. Exposure to conventional petroleum diesel EN590 exhaust (B0); 3. Exposure to biodiesel EN 14214 exhaust, blended with 30% conventional petroleum diesel (B30). PM (B0/B30) was collected and analyzed as described before [16] by the Netherlands Organization for Applied Scientific Research (TNO, The Netherlands). Mice were weight and anesthetized with an intraperitoneal injection of 75 mg/kg ketamine (Nimatek, Auv Cuijk, The Netherlands) and 3 mg/kg xylazine (Sedamun, Auv Cuijk, The Netherlands). The volume of 50 µL PM solution or saline (final concentrations in solution; B0: 3.6 µg/mL and B30: 2.06 µg/mL) was delivered in the trachea of each mouse using a Hamilton syringe. The concentrations B0 and B30 PM are representative for the amount of emission originating from the performed test cycle. After intratracheal instillation, mice were kept in an upright position for 10 min to allow the fluid to spread throughout the lungs. Subsequently, all animals were monitored until complete recovery. Several mice (n=3 from B0 group; n=2 from B30 group) were sacrificed before the end of experiments due to acute respiratory distress and were excluded from the study. The study design is described schematically in Figure 6.1.

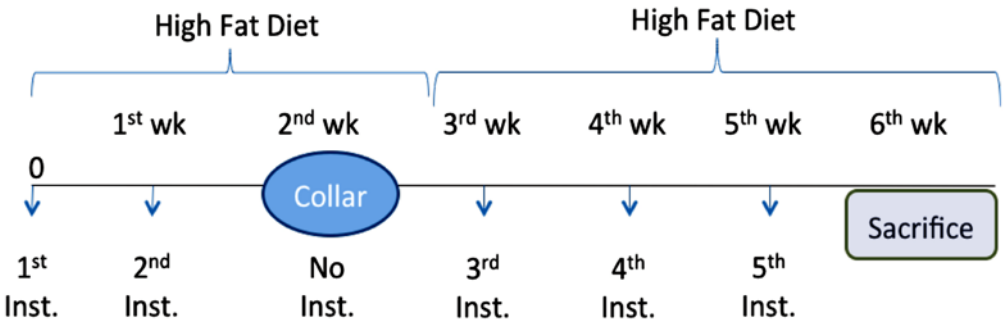


Figure 6.1. Design of the experiment
LDLR $^{-/-}$ mice received a collar placement around both common carotid arteries at week 2 in the course of an 8-week high-fat diet regimen. In addition, all mice were intratracheally instilled with 50 µL saline (n=9), PM Bo (n=6) or PM B30 (n=7) on a weekly basis. No instillation was carried out at week 2 due to the performed cuff placement surgery, thereby allowing better recovery for the animals. Mice were sacrificed at week 6 after collar placement for analysis of the atherosclerotic burden.

Tissue preparation and quantification

Mice were exsanguinated and perfused with sodium nitroprusside for 5 minutes (Sigma-Aldrich St. Louis, MO, USA; dissolved in phosphate-buffered saline (PBS), pH 7.4). Carotid arteries were fixed in 1 %(v/v) paraformaldehyde solution, embedded and cross-sectioned at 4 µm thickness. General plaque parameters were quantified using 6 consecutive hematoxylin and eosin (H&E)-stained sections, with 100 µm intervals. Vascular smooth muscle cell (VSMC) content was analyzed semi-quantitatively using the following scoring system: 0% cells stained = 0; 1-25% = 1; 26-50% = 2; 51-75% = 3 and 76-100% = 4. All quantification analyses were performed with Image-Pro Plus 7.0.1, Media Cybernetics Inc., Bethesda, MD, USA and Adobe Photoshop CS5 Extended, Adobe Systems, San Jose, CA, USA. The assessing investigator was blinded as to the specific group assignments.

Histology and immunohistochemistry

Cross carotid artery sections were stained with H&E and toluidine blue to evaluate main plaque characteristics. Sirius red was used to assess the relative collagen deposition in the atherosclerotic plaques. Sections were immunolabeled with the following antibodies: rabbit polyclonal to α-smooth muscle actin (1:100, Abcam, #ab5694) to detect vascular smooth muscle cells; rabbit polyclonal to anti-cleaved caspase-3 (1:100, Cell Signalling Technology, #N.9661) as a marker for apoptosis; mouse monoclonal to 8-hydroxy-2'deoxyguanosine (1:500,

Abcam, #ab26842) detecting DNA oxidant damage; rat polyclonal to Mac-3 (1:20, BD Pharmingen, #550292) as a marker for monocyte infiltration. Secondary biotinylated polyclonal rabbit anti-rat (1:100, Santa Cruz, #sc-2041), sheep anti- mouse Biotin (1:100, Amersham, #RPN1001) and polyclonal swine anti-rabbit (1:500, DakoCytomation Denmark A/S, Glostrup, Denmark, #E0431) immunoglobulins were used. A tertiary antibody streptavidin-alkaline Phosphatase (1:200, DakoCytomation Denmark A/S, #D0396) was applied and then followed by incubation with Vector Red (Vector Laboratories; Peterborough, UK). Hematoxylin staining was performed as a counterstain.

Blood sampling, blood cell counts and lipid profile analysis

Peripheral blood samples were collected in 3.2 %(w/v) sodium citrate through vena puncture (vena cava inferior) as described previously [17]. Blood cell counts were assessed using an automated hematology analyzer (Beckman Coulter, FL, USA). Blood samples were centrifuged for 15 minutes at 4500 rpm at RT. Plasma was transferred, centrifuged for 5 minutes at 14000 rpm, and stored at -80 °C. Using automated enzymatic colorimetric assays, we determined plasma total cholesterol, triglycerides and HDL and LDL (CHOD-PAP/GPO-PAP, Roche Diagnostics, Germany).

Statistical analysis

Statistical analyses were performed using Prism, version 5.00 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were tested with Mann-Whitney U test and data are expressed as median (IQR 25%-75%). A 2-tailed $p<0.05$ was considered statistically significant.

Results

Exposure to B0 and B30 does not affect atherosclerosis plaque growth

We analyzed the extent of atherosclerosis progression at week 6 after carotid collar placement in $LDLR^{-/-}$ mice, which were fed on a high-cholesterol regimen for 8 weeks, and exposed to petroleum diesel (B0) exhaust particles, biodiesel (B30) exhaust particles, or saline through an intratracheal instillation once per week. Exposure to B0 or B30 did not result in an accelerated

pro-atherogenic response and the extent of atherosclerotic plaque burden was comparable between animals exposed to saline and Bo or B30 (Fig. 6.2*A, B*; Saline: 6.7 (2.1-30.3) x10^3 µm^2 vs. Bo: 9.3 (4.8-27.0) x10^3 µm^2 vs. B30: 5.0 (3.7-26.7) x10^3 µm^2). There was no significant change in the degree of stenosis, intima-media ratio and total vessel lumen between Bo- or B30-treated and control mice (Fig. 6.2*C, D, E*). Total cholesterol, low- and high-density lipoprotein, triglyceride levels, and body weight, did not differ between the groups (*data not shown*).

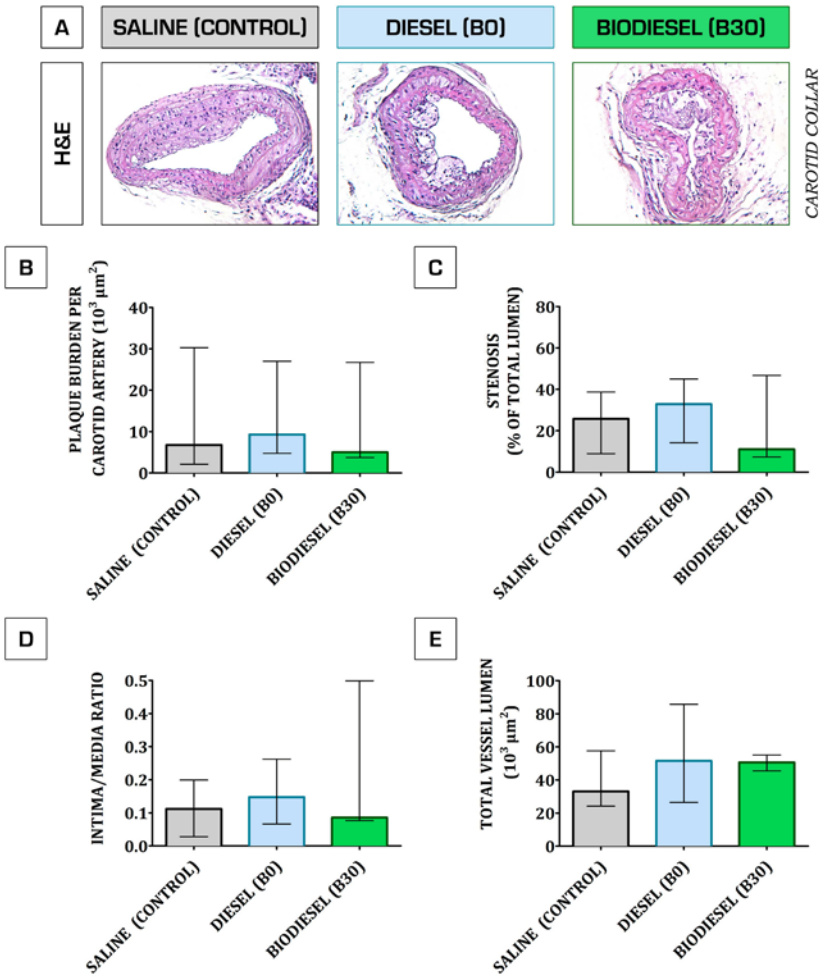


Figure 6.2. ***Carotid Atherosclerosis – Quantitative Analysis***
Sections of the carotid arteries were stained with hematoxylin and eosin (H&E) to analyze the extent of atherosclerosis plaque formation (panel A). Atherosclerotic plaque volume (panel B), degree of stenosis (% of the total vessel lumen; panel C), intima/media ratio (panel D) and total vessel lumen area (panel E) were not affected by the exposure to conventional diesel exhaust Bo (n=6; blue bars) or biodiesel exhaust B30 (n=7, green bars) and were not statistically different compared to LDLR$^{-/-}$ mice treated with saline (n=9, grey bars). Bars represent median (IQR 25%-75%).

Decreased plaque stability due to diminished total collagen content in biodiesel-instilled LDLR$^{-/-}$ mice

Given the pivotal role of monocytes and inflammation in all stages of atherosclerosis development [2], we performed an immunohistochemical analysis to determine the level of macrophage infiltration within the atherosclerotic lesions. There was no significant difference in the absolute number of Mac-3-positive cells per plaque between B0, B30 and saline-exposed mice (Fig. 6.3A – upper panel, B; Saline: 44.0 (10.0-63.5) vs. B0: 21.0 (11.0-66.0) vs. B30: 18.5 (12.3-28.8) Mac3-positive cells per plaque). Using Sirius Red staining and bright-field polarization microscopy, we determined the total collagen content as percentage of plaque area (Fig. 6.3A – lower panel, C). Atherosclerotic lesions in the saline treated group were characterized by 13% (6.3-30.9) collagen-positive areas and collagen deposition in biodiesel exhaust (B30) treated animals was significantly decreased (0.0% (0.0-2.9), p=0.03). Although not significantly, conventional diesel exhaust (B0) exposure diminished collagen deposition in LDLR$^{-/-}$ mice (0.4% (0.0-3.4), p=0.09).

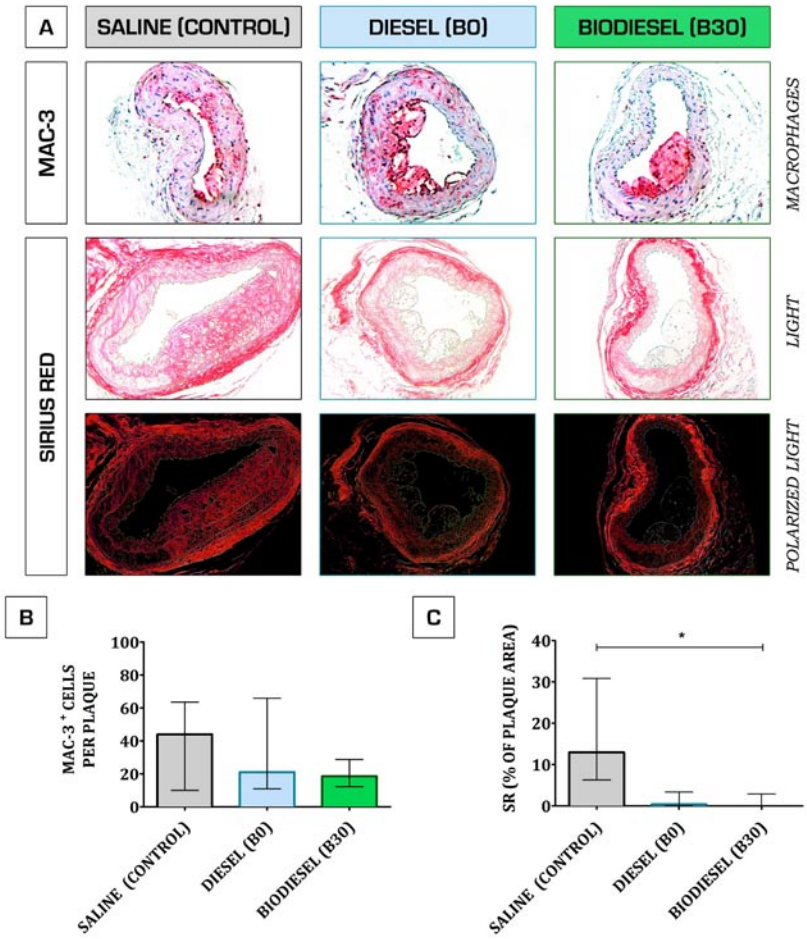


Figure 6.3. ***Atherosclerotic Plaque Composition – Macrophage infiltration and collagen content.*** Immunohistochemical analysis indicated no significant difference in the accumulation of Mac-3-positive cells within the atherosclerotic lesions of $LDLR^{-/-}$ mice instilled with conventional diesel exhaust (B0, n=6; blue bars), biodiesel exhaust (B30, n=7; green bars) and saline (control, n=9; grey bars) (panel A, upper part and panel B). Conventional diesel exhaust (B0) and biodiesel exhaust B30 treatment induced a decrease in atherosclerotic plaque collagen content with only a significant difference between saline and B30 treated animals (panel B, lower part and panel C) Bars represent median (IQR 25%-75%). * indicates $p<0.05$.

Exposure to biodiesel (B30) promotes plaque necrosis and fibrous cap thinning

We established the size of the necrotic core area in all experimental groups. Exposure to B30 resulted in significantly increased necrotic core volumes (37.5% (26.5-61.5) of total plaque area, p=0.03) compared to control mice (11.1% (10.1-22.2) of total plaque area), whereas no significant changes were observed after exposure to conventional diesel exhaust (B0: 28.9% (22.6-33.5) of total plaque area) (Fig. 6.4A – upper panel, B). In concordance with the collagen plaque content, we found a significant difference in the fibrotic appearance of the lesions in biodiesel exhaust (B30) treated mice, also in terms of fibrous cap thinning. Staining for α-SMA (smooth muscle cell actin) indicated that biodiesel exhaust (B30) exposure induced pronouncedly thinner fibrous caps (3.9 (3.1-4.3) μm, p=0.03) and reduced intimal VSMC content in comparison to saline treated control mice (10.7 (4.9-11.4) μm, Fig. 6.4A – lower panel, C). The mean fibrous cap thickness in the diesel exhaust (B0) treated animals was 4.3 (3.2-6.4) μm, but not statistically significant as compared to saline-instilled mice.

Number of detected advanced lesions in the control group was 3 (n=9), whereas it was 5 (n=7) in B30 group and 3 (n=6) in the B0 arm of the study (Fig. 6.4A – upper panel).

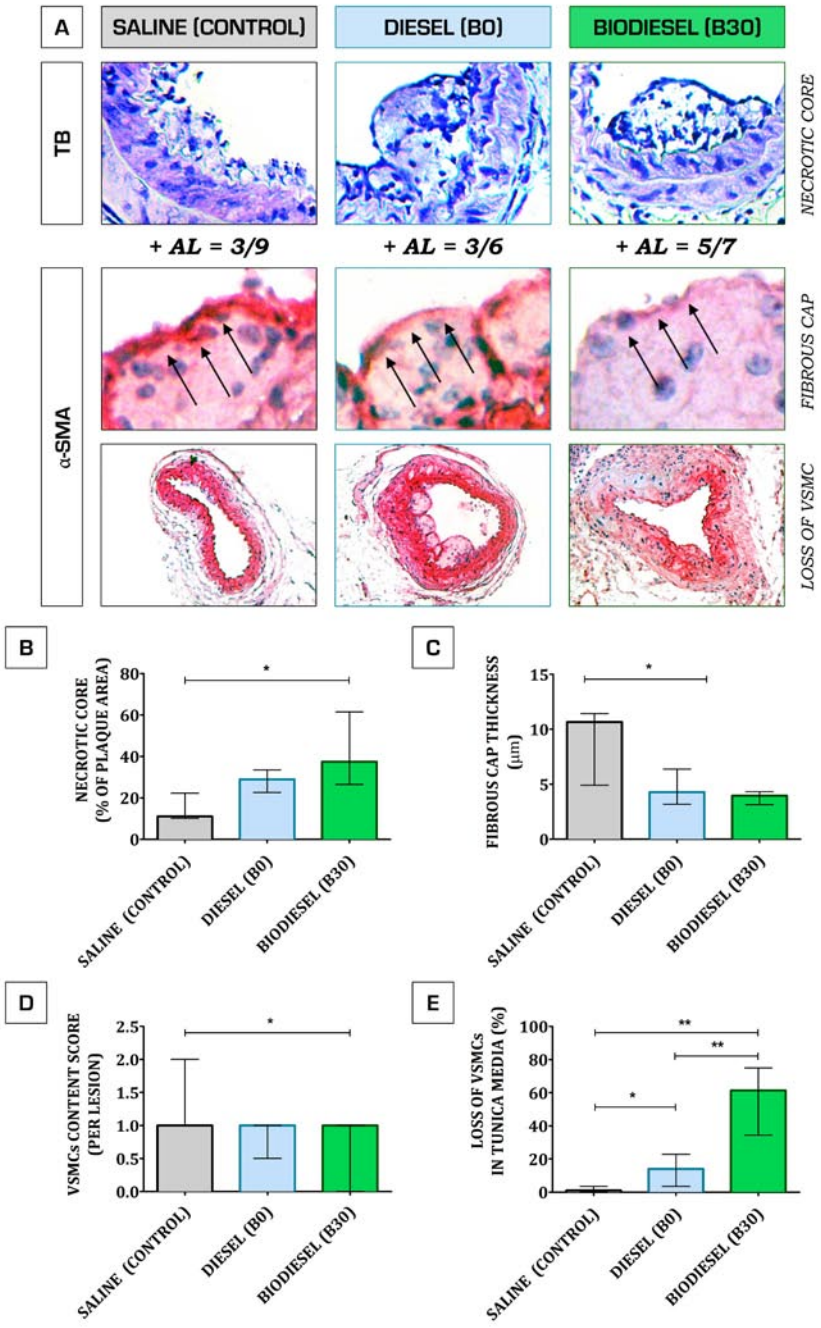


Figure 6.4. ***Atherosclerotic Plaque Composition – Necrotic Core Formation, Fibrous Cap Thickness and Vascular Smooth Muscle Cell (VSMC) Content***

Panel A (upper row) displays the extent of necrotic cores, assessed by toluidine blue staining. The number of advanced lesion per study arm are denoted below and indicated as advance lesion (AL). The lower rows of Panel A demonstrate α-SMA (smooth muscle cell actin) staining, performed to identify potential loss of VSMC in the arterial vessel wall, but also to provide more understanding of the fibrotic appearance and characteristics of the fibrous caps of the lesions. Quantification data of necrotic cores as percentage of total plaque area are presented in panel B, the thickness of the fibrous cap in panel C, vascular smooth muscle cell (VSMC) score of tunica intima in Panel D, loss of VSMCs as percentage of total tunica media area in panel E, with saline treated animals represented by grey bars, mice exposed to conventional diesel exhaust (B0) by blue bars and to biodiesel exhaust (B30) by green bars. Bars represent median (IQR25%-75%). * indicates $p<0.05$; ** indicates $P<0.01$.

Biodiesel (B30) triggers accelerated oxidant DNA damage and apoptosis, leading to VSMC loss in tunica media and atherosclerotic plaque instability

Previous animal studies have clearly documented an increased oxidative stress within atherosclerotic lesions as a result of exposure to conventional petroleum diesel exhaust [14]. α-SMA staining revealed a significant increase in the loss of vascular smooth muscle cells in tunica media in both B0- and B30-instilled mice (Fig. 6.4A – lower panel, E). Hence, to elucidate the effects of exposure to biodiesel on reactive oxygen species formation and apoptosis upon atherosclerosis progression, we further investigated the plaque phenotype by staining for oxidative stress-induced DNA damage and apoptosis. The levels of DNA damage (presented as 8-OH-dG-postive area) were significantly higher in atherosclerotic lesions of mice exposed to both biodiesel exhaust (B30: 3.9 (1.6-26.2) $x10^3$ μm^2) and diesel exhaust (B0: 4.0 (0.7-12.2) $x10^3$ μm^2) compared to saline (0.0 (0.0-1.1) $x10^3$ μm^2, p=0.01; Fig. 6.5A – upper panel, B). In addition, exposure to biodiesel exhaust (B30) revealed a substantial degree of apoptosis (Fig. 6.5A - lower panel, C) with a caspase-3 positive area of 57.7% (26.8-72.7) of the total plaque area compared to 10.5% (1.0-18.2) after exposure to conventional diesel exhaust (B0) and 5.0% (1.6-15.7) for saline treated mice, respectively. Of interest, a strong association was observed between the loss of VSMC and the apoptosis index in the vessel wall (Pearson $r=0.7$, $p<0.05$) (Fig. 6.5D).

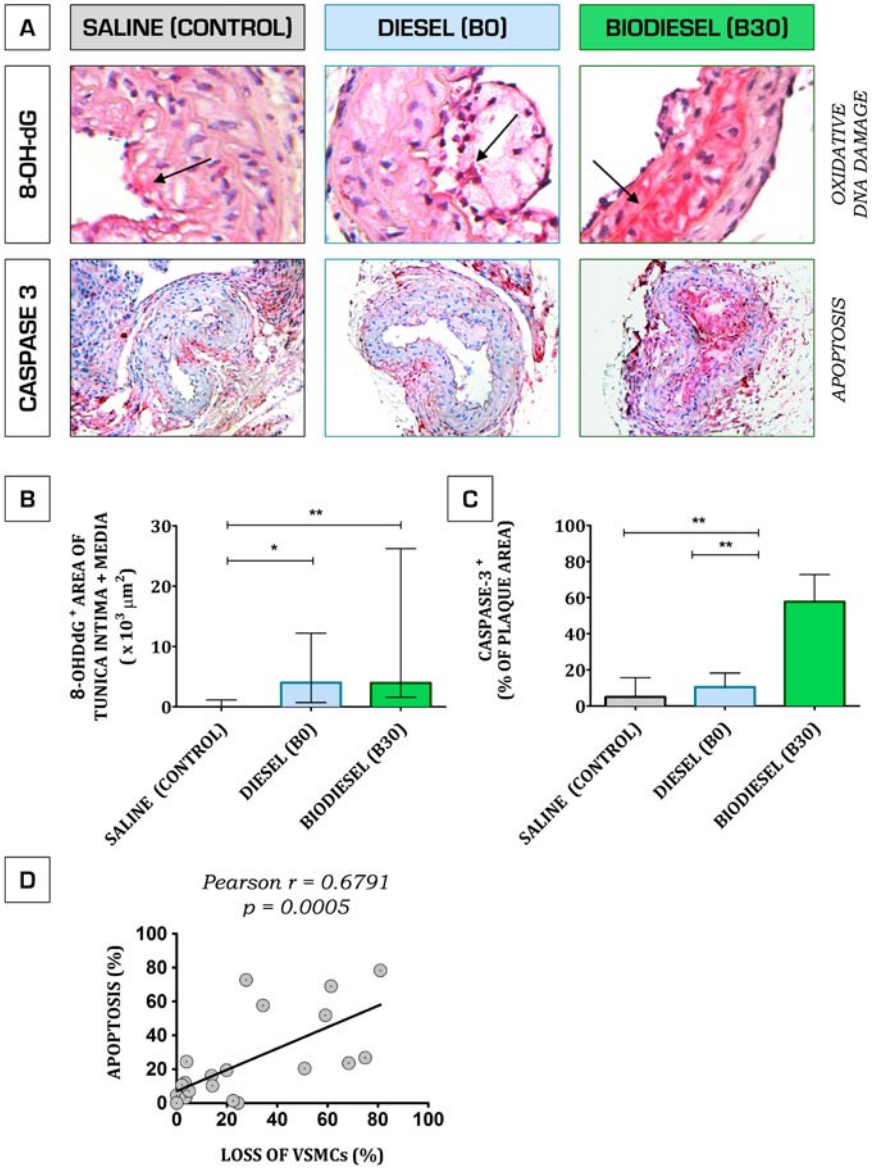


Figure 6.5. ***Enhanced Oxidant DNA Damage and Apoptosis Promote Unstable Plaque Phenotype in B30-instilled LDLR$^{-/-}$ mice***

Panel A shows both 8-OH-dG (upper row) and Caspase-3 (lower row) stainings, used as specific markers markers to detect oxidant DNA damage and apoptosis, respectively. Quantification of oxidant DNA damage (8-OH-dG positive) and apoptosis (caspase-3 positive) is presented in panels B and C, respectively. Saline treated animals are represented by grey bars, mice exposed to conventional diesel exhaust (B0) by blue bars and to biodiesel exhaust (B30) by green bars. Bars represent median (IQR 25%-75%). * indicates p<0.05; ** indicates P<0.01.

Discussion

In the present study we demonstrate that PM derived from both conventional petroleum diesel and biodiesel do not exacerbate atherosclerosis growth in terms of extent of plaque burden or degree of stenosis, however, biodiesel clearly affect the atherosclerotic plaque composition inducing a vulnerable lesion phenotype. We provide new data suggesting that biodiesel particulates, similar to diesel-derived ones, promote oxidative DNA damage in the arterial bed. In addition, our data indicate that exposure to biodiesel B30 exhaust substantially enhances apoptosis through caspase-3 activation, which is linked to a significant loss of VSMC (formation of extensive "grave yards" in tunica media), but also to an enhanced plaque necrosis, fibrous cap thinning and diminished total collagen content. Overall, despite its improved emission and proposed superior health profile, these novel data suggest that exposure to biodiesel exhaust has potentially hazardous effects on the cardiovascular system and promotes cytotoxic and plaque destabilizing mechanisms, ultimately resulting in a high-risk lesion phenotype.

Some of the observed phenomena might be in part explained by a recent study in which the relative cytotoxicity for different type of fuels and blends was tested [16]. Surprisingly, when the biodiesel percentage in blends was increased, a significant induction in the relative cytotoxicity was observed. Pure biodiesel (B100) extracts resulted in approximately 40% increase in the cytotoxic effects when compared to a positive control. Although there is only limited amount of literature published on this topic, our observations are comparable to the findings, which have already been established in studies carried out with conventional petroleum diesel exhaust. There are multiple cellular and molecular routes through which PM can exert its pro-atherogenic and plaque destabilizing actions. It is widely accepted that PM alters the cellular mitochondrial function, leading to increased reactive oxygen species (ROS) formation, ultimately resulting in oxidative damage to lipoproteins [18, 19] and DNA [20]. PM inhalation accelerates systemic inflammation by inducing the translocation of ROS and IL-6 from the lungs to the circulation [21, 22]. Human and experimental studies document a dose-dependent increase in oxidative stress-induced DNA damage following exposure to fine and ultrafine PM [23-25]. PM has a detrimental impact on the endothelium causing a persistent endothelial dysfunction, linked to numerous pro-atherogenic effects such as overexpression of monocyte chemoattractant protein-1 (MCP-1) and diminished endothelial nitric oxide (NO) synthase production [26, 27]. Furthermore, there is abundant scientific evidence consolidating the pro-apoptotic properties of PM, and supporting the involvement of the intrinsic apoptotic pathway [28]. Because of the extensive activation of Caspase-3, which we observe in the lesions

of the $LDLR^{-/-}$ mice exposed to biodiesel exhaust, one may assume that the extrinsic apoptotic pathway may be also part of the PM-mediated pro-apoptotic mechanisms. Certainly, additional studies are needed to provide more understanding into the mechanistic basis of these effects.

Another aspect, which may be potentially relevant to PM-induced atherosclerotic plaque vulnerability, involves the pro-coagulant properties of PM. A close interaction between coagulation and inflammation exists in atherogenesis, which may support modulation processes involved in the determination of the atherosclerotic plaque stability [29]. Exposure to PM is known to trigger a hypercoagulable state in both rodents and humans through suppression of the activity of key natural anti-coagulants and activation of both the extrinsic and intrinsic coagulation pathways [30-35]. Hence, exposure to PM may hypothetically impact atherosclerosis progression trough coagulation activation.

Our study has several limitations. First, intratracheal instillation may not replicate a true long-term inhalation exposure to PM. Second, in the present study we used a carotid cuff model of atherosclerosis, which provides information on atherosclerotic burden in carotid arteries only, whereas the effects on other susceptible arterial sites remain unclear. Third, we tested only one biodiesel blend B30 (30% biodiesel).

In conclusion, the data presented in this study imply that exposure to biodiesel exhaust may impact atherosclerotic plaque progression, leading to unstable plaque phenotype through the activation of various pro-oxidant and pro-apoptotic mechanisms. Additional research is needed to further elucidate the molecular pathways involved.

Acknowledgements

Evren Kilinç is sponsored by the Netherlands Heart Foundation (grant number 2006B064) and Dr. J.I. Borissoff is a recipient of a Kootstra Talent Fellowship (2011) from Maastricht University and is supported by a Rubicon fellowship, granted by the Netherlands Organization for Scientific Research (NWO).

References

1 Brook RD, Rajagopalan S, Pope CA, 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC, Jr., Whitsel L, Kaufman JD. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation*. 2010; **121**: 2331-78.

2 Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999; **340**: 115-26.

3 Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, van Eeden SF. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol*. 2002; **39**: 935-42.

4 Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *Jama*. 2005; **294**: 3003-10.

5 Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ, Nel AE. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circ Res*. 2008; **102**: 589-96.

6 Kunzli N, Jerrett M, Mack WJ, Beckerman B, LaBree L, Gilliland F, Thomas D, Peters J, Hodis HN. Ambient air pollution and atherosclerosis in Los Angeles. *Environ Health Perspect*. 2005; **113**: 201-6.

7 Hoffmann B, Moebus S, Mohlenkamp S, Stang A, Lehmann N, Dragano N, Schmermund A, Memmesheimer M, Mann K, Erbel R, Jockel KH. Residential exposure to traffic is associated with coronary atherosclerosis. *Circulation*. 2007; **116**: 489-96.

8 Kunzli N, Jerrett M, Garcia-Esteban R, Basagana X, Beckermann B, Gilliland F, Medina M, Peters J, Hodis HN, Mack WJ. Ambient air pollution and the progression of atherosclerosis in adults. *PLoS One*. 2010; **5**: e9096.

9 Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. *Circulation*. 2001; **103**: 2810-5.

10 Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, Lowel H. Exposure to traffic and the onset of myocardial infarction. *N Engl J Med*. 2004; **351**: 1721-30.

11 Pope CA, 3rd, Burnett RT, Thurston GD, Thun MJ, Calle EE, Krewski D, Godleski JJ. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation*. 2004; **109**: 71-7.

12 Miller KA, Siscovick DS, Sheppard L, Shepherd K, Sullivan JH, Anderson GL, Kaufman JD. Long-term exposure to air pollution and incidence of cardiovascular events in women. *N Engl J Med*. 2007; **356**: 447-58.

13 Swanson KJ, Madden MC, Ghio AJ. Biodiesel exhaust: the need for health effects research. *Environ Health Perspect*. 2007; **115**: 496-9.

14 Bai N, Kido T, Suzuki H, Yang G, Kavanagh TJ, Kaufman JD, Rosenfeld ME, van Breemen C, Eeden SF. Changes in atherosclerotic plaques induced by inhalation of diesel exhaust. *Atherosclerosis*. 2011; **216**: 299-306.

15 von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation*. 2001; **103**: 1164-70.

16 Kooter IM, van Vugt M, Jedynska AD, Tromp PC, Houtzager MMG, Verbeek RP, Kadijk G, Mulderij M, Krul CAM. Toxicological characterization of diesel engine emissions using biodiesel and a closed soot filter. *Atmos Environ*. 2011; **45**: 1574-80.

17 Sommeijer DW, van Oerle R, Reitsma PH, Timmerman JJ, Meijers JC, Spronk HM, ten Cate H. Analysis of blood coagulation in mice: pre-analytical conditions and evaluation of a home-made assay for thrombin-antithrombin complexes. *Thromb J*. 2005; **3**: 12.

18 Jacobs L, Emmerechts J, Hoylaerts MF, Mathieu C, Hoet PH, Nemery B, Nawrot TS. Traffic air pollution and oxidized LDL. *PLoS One*. 2011; **6**: e16200.

19 Soares SR, Carvalho-Oliveira R, Ramos-Sanchez E, Catanozi S, da Silva LF, Mauad T, Gidlund M, Goto H, Garcia ML. Air pollution and antibodies against modified lipoproteins are associated with atherosclerosis and vascular remodeling in hyperlipemic mice. *Atherosclerosis*. 2009; **207**: 368-73.

20 Moller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, Vesterdal LK, Forchhammer L, Wallin H, Loft S. Role of oxidative damage in toxicity of particulates. *Free Radic Res*. 2010; **44**: 1-46.

21 Kido T, Tamagawa E, Bai N, Suda K, Yang HH, Li Y, Chiang G, Yatera K, Mukae H, Sin DD, Van Eeden SF. Particulate matter induces translocation of IL-6 from the lung to the systemic circulation. *Am J Respir Cell Mol Biol*. 2011; **44**: 197-204.

22 Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environ Health Perspect*. 2002; **110**: 749-55.

23 Kim JY, Mukherjee S, Ngo LC, Christiani DC. Urinary 8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to fine particulates. *Environ Health Perspect*. 2004; **112**: 666-71.

24 Tokiwa H, Sera N, Nakanishi Y, Sagai M. 8-Hydroxyguanosine formed in human lung tissues and the association with diesel exhaust particles. *Free Radic Biol Med*. 1999; **27**: 1251-8.

25 Ichinose T, Yajima Y, Nagashima M, Takenoshita S, Nagamachi Y, Sagai M. Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis*. 1997; **18**: 185-92.

26 Mo Y, Wan R, Chien S, Tollerud DJ, Zhang Q. Activation of endothelial cells after exposure to ambient ultrafine particles: the role of NADPH oxidase. *Toxicol Appl Pharmacol*. 2009; **236**: 183-93.

27 Yamawaki H, Iwai N. Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. *Circ J*. 2006; **70**: 129-40.

28 Urich D, Soberanes S, Burgess Z, Chiarella SE, Ghio AJ, Ridge KM, Kamp DW, Chandel NS, Mutlu GM, Budinger GR. Proapoptotic Noxa is required for particulate matter-induced cell death and lung inflammation. *Faseb J*. 2009; **23**: 2055-64.

29 Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med*. 2011; **364**: 1746-60.

30 Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandstrom T, Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation*. 2005; **112**: 3930-6.

31 Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest*. 2007; **117**: 2952-61.

32 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.

33 Lippi G, Favaloro EJ, Franchini M, Guidi GC. Air pollution and coagulation testing: a new source of biological variability? *Thromb Res*. 2008; **123**: 50-4.
34 Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. Diesel exhaust inhalation increases thrombus formation in man. *Eur Heart J*. 2008; **29**: 3043-51.
35 Kilinc E, Van Oerle R, Borissoff JI, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HM. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *J Thromb Haemost*. 2011; **9**: 1359-67.

CHAPTER 7

GENERAL DISCUSSION

Air pollution is a complex mixture of compounds in gas (ozone (O_3), carbon monoxide (CO) and nitrogen oxide (NO), nitrogen dioxide (NO_2)) and particle phases; the strongest evidence among several epidemiological studies linking air pollution with human health effects centers around the particulate components [1-7].

Particulate matter (PM) is comprised of heterogenous particles varying in size, number, chemical composition, concentration and source [2] and includes the particles that are emitted from sources such as fossil-fuel combustion (e.g. diesel exhaust particles). Particles are classified according to their aerodynamic diameter into size fractions such as PM10 (< 10 μm), PM2.5 (< 2.5 μm) and ultrafine particles (UFPs; < 0.1 μm). Inhaled particles end up in various segments of the human respiratory tract. While the larger PM10 particles impact on the tracheal region, the smaller PM2.5 particles penetrate deeper into the bronchi and bronchioli, whereas the UFPs reach the alveolar regions.

Increase in ambient PM10 and PM2.5 concentrations is associated with an increased risk of stroke, myocardial ischemia, and coronary artery disease [8-14]. Although the studies with PM10 and PM2.5 data suggest that smaller sized particles are related to larger cardiovascular effects, there are only few reports supporting the association of UFPs with increased mortality [15, 16]. Blood coagulation plays an important role in cardiovascular disease since several coagulation factors have been recognized as risk factors for thrombosis. Indeed, PM10 inhalation was identified as a risk factor for venous thromboembolism [17-19]. Additionally, PM exposure can induce changes in numerous proteins associated with inflammation and coagulation, including increased C-reactive protein (CRP) [20-22], plasminogen activator inhibitor-1 (PAI-1) [20, 23, 24], fibrinogen [20, 24-26] and von Willebrand factor (vWF) [22]. However, no association between PM exposure and pro-thrombotic effects or haemostatic alterations has been found [27-31].

Two distinct pathways by which PM modulates its hazardous effects have been proposed: one route in which pulmonary inflammation is the initiating mechanism, another route by which direct translocation of UFPs into the systemic circulation is the main pathway.

Exposure to PM induces a pro-inflammatory response in human lungs [25] consistent with observations in *in vivo* animal models [32, 33]. The PM-induced pulmonary inflammation is followed by the release of inflammatory cytokines, such as interleukin (IL)-1β and IL-6 [34] in the circulation, resulting in the release of bone-marrow derived neutrophils and monocytes [35]. The generation of a systemic inflammatory response has been documented by increases in CRP [36, 37], potentially stimulating coagulation activity. Additionally, knock-out mice that lacked IL-6 were protected against the prothrombotic effects of PM exposure [19].

Increasing evidence points to an extensive cross-talk between inflammation and hemostasis, whereby inflammation leads to activation of platelets and coagulation, while activated blood platelets and coagulation factors also contribute to the inflammatory action [38]. This way, pulmonary inflammation induced by PM exposure may elicit a chain of ultimately prothrombotic effects.

Direct procoagulant effects may occur via UFPs that cross the pulmonary epithelial barrier. This systemic translocation of particles was demonstrated in experimental animal models [39, 40]. In contrast, the evidence of systemic translocation from human studies is less clear, with both positive [41] and negative [42] findings. Once UFPs have translocated to the blood circulation, they can be distributed throughout the body, interacting with the vascular endothelium or circulating cells, and proteins. Given the role of platelets in hemostasis, obviously, the PM-mediated effects on platelets are modulated either directly by UFPs after translocation into blood [43-45], or indirectly via secondary mediators such as tissue factor (TF) bearing microparticles.

In relation to cardiovascular disease, several human and animal studies have shown that exposure to PM induces progression of atherosclerosis [46-53]. It is likely that PM induced atherosclerosis, either driven by pulmonary and ultimately systemic inflammation, or by UFPs directly affecting several pro-atherogenic processes. Experimental evidence implies that NOXA (p53-upregulated modulator of apoptosis, intrinsic apoptotic pathway) dependent apoptosis might modulate the release of IL-6 by alveolar macrophages however, the precise mechanisms by which this occurs is not clear[54, 55]. It is plausible that phagocytosis of the particles by pulmonary macrophages induces ROS production and afterwards cell apoptosis. In vascular cells, apoptosis contributes to atherosclerotic plaque growth, lipid core development, plaque rupture and thrombosis but the extent to which apoptosis regulates these processes is unknown [56]. At an early stage of atherosclerosis, oxidation of low density lipoprotein (ox-LDL) leading to foam cell formation, is crucial. Endothelial dysfunction (possibly induced by PM driven systemic inflammation [57, 58] or directly by UFPs [59]) promotes accumulation of ox-LDL and inflammatory cell infiltration especially monocytes, in the vasculature. Increased plasma ox-LDL upon exposure to PM has been suggested by several experimental studies [60-62]. Formation of foam cells exacerbates the formation of atherosclerotic plaque by several inflammatory processes such as secretion of proinflammatory cytokines. ROS production by foam cells enhances leukocyte chemotaxis through vascular endothelium and also regulates vascular smooth muscle cell (VSMC) migration and proliferation into the intima. Ox-LDL itself may induce the expression of

adhesion molecules such as vascular cell adhesion protein- 1 (VCAM-1), inter-cellular adhesion molecule-1 (ICAM-1) and P-selectin, scavenger receptors and matrix metalloproteinases (MMPs). In addition, ox-LDL triggers migration and apoptosis of VSMCs [63, 64]. Although the human studies center around PM2.5 induced atherosclerosis, animal studies suggest that smaller sized particles are related to larger atherosclerotic plaques. Under comparable experimental conditions, UFPs induced thicker atherosclerotic plaques than PM2.5 in Apo E -/- mice [65].

In this thesis, we focused on the effects of particles of different size on hemostasis and inflammation. Furthermore, it brings a mechanistic understanding for cardiovascular effects of air pollution since the studies focus on hemostasis, thrombosis and atherosclerosis.

In **chapter 2**, we investigated the associations between air pollution and platelet aggregation, thrombin generation (TF dependent) and inflammation in healthy human subjects. PM10, CO, NO, NO_2 and O_3 were associated with increased platelet aggregation and only gas pollutants, but not PM10, were associated with increased thrombin generation (triggered with 1 pM TF). Additionally, the inflammatory markers fibrinogen and CRP did not show any significant association with air pollution. The significant associations within 24–96 h before blood sampling suggest that exposure to air pollution indirectly increases blood thrombogenicity. These indirect effects may be the result of air pollution–induced synthesis of TF [66], which can increase *in vivo* platelet reactivity [67]. It is known that gas pollutants can be considered markers for motor vehicle traffic and have been shown to be highly correlated with PM2.5 and UFPs [68]. Thus, it is mainly this subset (especially UFPs) from the overall PM air pollution that has an effect on thrombin generation as shown in **chapter 4**. In our studies, we did not specifically address the question of inflammation mediated pro-coagulant activity. However, this interaction has been explored by others, showing that changes in the hemostatic balance can be inflammation-driven, showing IL-6 and tumor necrosis factor - alpha (TNF-α) regulated pro-coagulant effects of PM. It has also been shown that phagocytosis of PM10 and PM2.5 by pulmonary macrophages induces an IL-6 dependent pro-coagulant response [19, 69].

In **chapter 3**, the pulmonary and cardiovascular effects of PM2.5 were explored. Exposure to diesel exhaust PM (PM_{DEE}) in Fischer rats for 4 weeks resulted in significantly reduced numbers of white blood cells (WBC), lymphocytes and basophilic granulocytes. On the other hand, PM2.5 roadside exposure did not induce any significant changes in blood parameters. Additionally, a reduction in vWF levels was observed 4-weeks after exposure to PM_{DEE}. Lung pathology and vascular function did not alter after exposure to PM_{DEE} and PM2.5 and

consistently, no significant changes were observed in mRNA expressions of IL-1β, IL-6 and TNF-α in heart tissue. Furthermore, changes in lung TF activity and thrombin generation parameters (lag time, endogenous thrombin potential (ETP) and peak height) were observed. Surprisingly, lung TF activity was decreased after both exposure to PM_{DEE} and PM2.5. Although no changes were found in lung thrombin generation in the presence of 5 pM TF, an unexpected significant decrease in lung thrombin generation was observed when triggered with only lung homogenates after exposure to PM_{DEE} and PM2.5.

These somewhat surprising results suggest that chronic exposure to PM may not give the same proinflammatory and procoagulant reactions as acute PM exposure and that different underlying mechanisms are effective.

General effects of PM in this study may be related to O3 exposure prior to the repeated non-continuously exposure to particulate matter for five days, followed by exposure to control air. Animals were exposed to O_3 prior to prolonged exposure to traffic PM, which was intended to cause significant pulmonary inflammation. O_3 is known to provoke damage of type I epithelial cells and increased permeability of the alveolar walls [70, 71]. Together with the repeated non-continuously exposure to PM, this may have caused a desensitizing effect on inflammation and coagulation. Another explanation for the decrease in lung-specific thrombin generation might be that thrombomodulin is not down regulated as would be the case in acute exposure [72], but rather upregulated upon continuously inflammation. Within atherosclerotic lesions, thrombomodulin is expressed by macrophages [73] and the presence of macrophages derived thrombomodulin within lung tissue could compensate for the decrease of endothelial cell expressed thrombomodulin. An increased anticoagulant response is obviously explained by decreased thrombin generation in the presence of only phospholipids whereas no difference was found with both 5 pM TF and phospholipids. Therefore, the balance in pro- and anticoagulant reactions to PM will modulate the net thrombotic events via thrombin. Finally, the potent suppression effects of PM_{DEE} compared to PM2.5 roadside exposures in this study may refer to UFPs concentration, which was substantially higher for the PM_{DEE}.

In **chapter 4**, the effects of PM on blood coagulation both *in vitro* and in mice was demonstrated. PM10, PM2.5 and UFPs induced thrombin generation in human plasma. However, upon addition of corn trypsin inhibitor (CTI) to plasma, PM failed to increase thrombin generation. Lacking thrombin generation in factor (F) XII and FXI deficient plasma after PM addition strongly argued for PM induced thrombin generation via activation of FXII, initiating the intrinsic pathway of coagulation. Additionally, PM induced FXII activation was

confirmed by a chromogenic activity measurement. In respect to the small size of UFPs, their translocation into the circulation may theoretically result in direct activation of blood coagulation proteins including FXII. Thus, UFPs and saline (control) were intratracheally administered to both wild type (WT) and factor XII deficient ($FXII^{-/-}$) mice. Blood samples were collected from mice at 4 and 20 hrs after administration of UFPs. A limited but not significant increase of TF dependent thrombin generation was observed in plasma of mice after 4 hrs. However, although there was no TF dependent thrombin generation in WT and $FXII^{-/-}$ mice plasma, FXII dependent thrombin generation in WT mice plasma was increased after 20 hrs of UFPs administration. The extended question how the late activation of FXII (at 20 hrs) occurs still remains to be further elucidated. It is possible that the delayed FXII activation is the result of protracted assembly of protein activating complexes on phospholipid sufaces provided by activated cells such as platelets or microparticles. In fact, FXII mediated contact activation by activated platelets and possibly through release of polyphosphates has been shown [74]. Experimental evidence also showed that FXII deficient mice were partly protected against arterial thrombosis [75]. Additionally, a recent study by our group elucidated the role of activated FXII in regulation of fibrin structure independently from thrombin generation [76]. Thus, the key findings of this study on PM mediated procoagulant activity may support a FXII mediated role in arterial thrombosis, hence in cardiovascular disease in broader sense.

We have taken our understanding of UFPs mediated blood coagulation a step further and investigated TF mediated thrombin generation in human volunteers after exposure to (i) dilute diesel exhaust, (ii) pure carbon nanoparticulate, (iii) filtered diesel exhaust, or (iv) filtered air, in a randomized double blind cross-over study. In **chapter 5**, the key findings are presented. Blood samples were collected after 2, 6 and 24 hrs and thrombin generation in human plasma was measured in the presence of 1 pM TF. The results reflect no significant changes in thrombin generation parameters after exposure to none of the particle or gas pollutants, in accordance with our early findings in mice presented in **chapter 4**. Based on the observations from an earlier study, exposure to DEE was found to significantly increase *ex vivo* arterial thrombus formation in a Badimon chamber compared to filtered air [77]. Additionally, a preferential effect of intra-tracheal PM on arterial rather than venous thrombosis in mice has also been suggested [32].

We revealed a contribution of the FXII dependent intrinsic pathway of coagulation to the *in vitro* but also *in vivo* procoagulant effects of PM. These data add to the accumulating evidence

for a certain contribution of FXII to cardiovascular disease/atherothrombosis. Although from a healthcare perspective these data may be of trivial importance, mechanistically it raises new avenues also for drug development (anti-FXII mediated anticoagulants).

A second important observation is that although acute PM exposures are usually prothrombotic, prolonged (repeated) exposure is not obviosuly prothrombotic, in contrast, all procoagulant effects appear to be down regulated. This is important in considering the strength of the effects of daily PM exposure (although likely unhealthy, it may be better tolerated than anticipated).

Third, in acute exposure studies there are marked differences in PM effects on thrombin generation, based on differences in chemical composition. This may indeed confirm that depending on the location, PM may be more or less harmful (if thrombin generation can be translated to cardiovascular risk, which is still speculative).

Growing concern regarding energy resources and the environment has increased interest in the study of alternative sources of energy. To meet increasing energy requirements, there is a growing interest in alternative fuels like biodiesel. Biodiesels offer a very promising alternative to diesel oil since they are renewable and have similar properties. The main advantage in biodiesel usage is attributed to lesser exhaust emissions in terms of carbon monoxide, hydrocarbons and particulate matter [78]. Thus, it can be expected that biodiesel has less cardiovascular hazard, because of a reduced particulate matter burden. However, there is only one published experimental study related to the proatherogenic effects of DEE [46], whereas no research has ever determined the potential differences in effects of biodiesel versus regular diesel exposure on the cardiovascular system, including atherosclerotic plaque development and plaque phenotype *in vivo*.

In **chapter 6**, $LDL^{-/-}$ mice were intratracheally instilled with comparable concentrations of diesel and biodiesel particles. We performed an extensive cross-sectional quantification of the common carotid arteries (proximal, intra-cuff and distal regions). There were no significant differences found in atherosclerotic plaque area, degree of stenosis and mean outer diameter between the intervention and control groups. In contrast, the morphologic characteristics of the lesions were substantially distinct between biodiesel PM-treated mice and the rest of the groups. Biodiesel PM exhibited an unstable plaque phenotype, characterized by large cellular necrotic cores, thin fibrous caps, decreased collagen content and loss of VSMC in the shoulder regions of the plaque. Our initial data show that biodiesel PM is more pro-oxidative compared to normal diesel PM, which may in part explain the increased apoptosis and cell death.

Overall, given the abundant evidence on the role of oxidative stress in atherogenesis, this data suggest that biodiesel PM may ultimately contribute to a higher risk for atherosclerosis. This observation underscores the need for further studies of so called “green” gasoline fractions like biodiesel.

FUTURE PLANS

The above discussion creates new avenues for study that appear relevant from a healthcare perspective.

First, it seems imperative from a toxicological perspective to get better insight in the differences in effects of acute versus chronic exposure to PM and related to chemical composition. This will need to be accompanied by a critical analysis of the different animal models used.

Second, from a societal perspective studies on biodiesel versus other gasolines, also including any protective effects of gasoline filters, remain important. It is surprising that such studies were not yet done in the first place, given the speed by which the government decided to proceed towards the development of biodiesel and other “green” gasoline applications.

From a mechanistic point and easier achievable, may be further studies on (development) of inhibitors of the contact activation and intrinsic systems. These studies are directly related to cardiovascular disease since intervention in these mechanisms may reduce arterial thrombosis associated organ damage. Development of contact system (factor XII) inhibitors is currently explored by pharmaceutical companies and testing of such compounds in suitable models for ischemic stroke and myocardial infarction seems a topic of interest.

References

1 Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation*. 2002; **105**: 1534-6.

2 Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*. 2004; **109**: 2655-71.

3 Brook RD, Rajagopalan S, Pope CA, 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC, Jr., Whitsel L, Kaufman JD. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation*. 2010; **121**: 2331-78.

4 Gold DR, Litonjua A, Schwartz J, Lovett E, Larson A, Nearing B, Allen G, Verrier M, Cherry R, Verrier R. Ambient pollution and heart rate variability. *Circulation*. 2000; **101**: 1267-73.

5 Ibald-Mulli A, Stieber J, Wichmann HE, Koenig W, Peters A. Effects of air pollution on blood pressure: a population-based approach. *Am J Public Health*. 2001; **91**: 571-7.

6 Peters A, Perz S, Doring A, Stieber J, Koenig W, Wichmann HE. Increases in heart rate during an air pollution episode. *Am J Epidemiol*. 1999; **150**: 1094-8.

7 Pope CA, 3rd, Verrier RL, Lovett EG, Larson AC, Raizenne ME, Kanner RE, Schwartz J, Villegas GM, Gold DR, Dockery DW. Heart rate variability associated with particulate air pollution. *Am Heart J*. 1999; **138**: 890-9.

8 Ballester F, Rodriguez P, Iniguez C, Saez M, Daponte A, Galan I, Taracido M, Arribas F, Bellido J, Cirarda FB, Canada A, Guillen JJ, Guillen-Grima F, Lopez E, Perez-Hoyos S, Lertxundi A, Toro S. Air pollution and cardiovascular admissions association in Spain: results within the EMECAS project. *J Epidemiol Community Health*. 2006; **60**: 328-36.

9 Kettunen J, Lanki T, Tiittanen P, Aalto PP, Koskentalo T, Kulmala M, Salomaa V, Pekkanen J. Associations of fine and ultrafine particulate air pollution with stroke mortality in an area of low air pollution levels. *Stroke*. 2007; **38**: 918-22.

10 Lanki T, Pekkanen J, Aalto P, Elosua R, Berglind N, D'Ippoliti D, Kulmala M, Nyberg F, Peters A, Picciotto S, Salomaa V, Sunyer J, Tiittanen P, von Klot S, Forastiere F. Associations of traffic related air pollutants with hospitalisation for first acute myocardial infarction: the HEAPSS study. *Occup Environ Med*. 2006; **63**: 844-51.

11 Pope CA, 3rd, Muhlestein JB, May HT, Renlund DG, Anderson JL, Horne BD. Ischemic heart disease events triggered by short-term exposure to fine particulate air pollution. *Circulation*. 2006; **114**: 2443-8.

12 Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med*. 2006; **173**: 432-41.

13 Seaton A, Soutar A, Crawford V, Elton R, McNerlan S, Cherrie J, Watt M, Agius R, Stout R. Particulate air pollution and the blood. *Thorax*. 1999; **54**: 1027-32.

14 Wellenius GA, Schwartz J, Mittleman MA. Air pollution and hospital admissions for ischemic and hemorrhagic stroke among medicare beneficiaries. *Stroke*. 2005; **36**: 2549-53.

15 Stolzel M, Breitner S, Cyrys J, Pitz M, Wolke G, Kreyling W, Heinrich J, Wichmann HE, Peters A. Daily mortality and particulate matter in different size classes in Erfurt, Germany. *J Expo Sci Environ Epidemiol*. 2007; **17**: 458-67.

16 Wichmann HE, Spix C, Tuch T, Wolke G, Peters A, Heinrich J, Kreyling WG, Heyder J. Daily mortality and fine and ultrafine particles in Erfurt, Germany part I: role of particle number and particle mass. *Res Rep Health Eff Inst*. 2000: 5-86; discussion 7-94.

17 Baccarelli A, Martinelli I, Zanobetti A, Grillo P, Hou LF, Bertazzi PA, Mannucci PM, Schwartz J. Exposure to particulate air pollution and risk of deep vein thrombosis. *Arch Intern Med*. 2008; **168**: 920-7.

18 Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost*. 2007; **5**: 252-60.

19 Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest*. 2007; **117**: 2952-61.

20 Chuang KJ, Chan CC, Su TC, Lee CT, Tang CS. The effect of urban air pollution on inflammation, oxidative stress, coagulation, and autonomic dysfunction in young adults. *Am J Respir Crit Care Med*. 2007; **176**: 370-6.

21 Delfino RJ, Staimer N, Tjoa T, Polidori A, Arhami M, Gillen DL, Kleinman MT, Vaziri ND, Longhurst J, Zaldivar F, Sioutas C. Circulating biomarkers of inflammation, antioxidant activity, and platelet activation are associated with primary combustion aerosols in subjects with coronary artery disease. *Environ Health Perspect*. 2008; **116**: 898-906.

22 Schicker B, Kuhn M, Fehr R, Asmis LM, Karagiannidis C, Reinhart WH. Particulate matter inhalation during hay storing activity induces systemic inflammation and platelet aggregation. *Eur J Appl Physiol*. 2009; **105**: 771-8.

23 Cozzi E, Wingard CJ, Cascio WE, Devlin RB, Miles JJ, Bofferding AR, Lust RM, Van Scott MR, Henriksen RA. Effect of ambient particulate matter exposure on hemostasis. *Transl Res*. 2007; **149**: 324-32.

24 Su TC, Chan CC, Liau CS, Lin LY, Kao HL, Chuang KJ. Urban air pollution increases plasma fibrinogen and plasminogen activator inhibitor-1 levels in susceptible patients. *Eur J Cardiovasc Prev Rehabil*. 2006; **13**: 849-52.

25 Ghio AJ, Kim C, Devlin RB. Concentrated ambient air particles induce mild pulmonary inflammation in healthy human volunteers. *Am J Respir Crit Care Med*. 2000; **162**: 981-8.

26 Ruckerl R, Greven S, Ljungman P, Aalto P, Antoniades C, Bellander T, Berglind N, Chrysohoou C, Forastiere F, Jacquemin B, von Klot S, Koenig W, Kuchenhoff H, Lanki T, Pekkanen J, Perucci CA, Schneider A, Sunyer J, Peters A, Group AS. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environ Health Perspect*. 2007; **115**: 1072-80.

27 Brauner EV, Moller P, Barregard L, Dragsted LO, Glasius M, Wahlin P, Vinzents P, Raaschou-Nielsen O, Loft S. Exposure to ambient concentrations of particulate air pollution does not influence vascular function or inflammatory pathways in young healthy individuals. *Part Fibre Toxicol*. 2008; **5**: 13.

28 Carlsten C, Kaufman JD, Peretz A, Trenga CA, Sheppard L, Sullivan JH. Coagulation markers in healthy human subjects exposed to diesel exhaust. *Thromb Res*. 2007; **120**: 849-55.

29 Forbes LJ, Patel MD, Rudnicka AR, Cook DG, Bush T, Stedman JR, Whincup PH, Strachan DP, Anderson RH. Chronic exposure to outdoor air pollution and markers of systemic inflammation. *Epidemiology*. 2009; **20**: 245-53.

30 Steinvil A, Kordova-Biezuner L, Shapira I, Berliner S, Rogowski O. Short-term exposure to air pollution and inflammation-sensitive biomarkers. *Environ Res*. 2008; **106**: 51-61.

31 Sullivan JH, Hubbard R, Liu SL, Shepherd K, Trenga CA, Koenig JQ, Chandler WL, Kaufman JD. A community study of the effect of particulate matter on blood measures of inflammation and thrombosis in an elderly population. *Environ Health*. 2007; **6**: 3.

32 Emmerechts J, Alfaro-Moreno E, Vanaudenaerde BM, Nemery B, Hoylaerts MF. Short-term exposure to particulate matter induces arterial but not venous thrombosis in healthy mice. *J Thromb Haemost*. 2010; **8**: 2651-61.

33 Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. *Am J Respir Crit Care Med*. 2003; **168**: 1366-72.

34 van Eeden SF, Tan WC, Suwa T, Mukae H, Terashima T, Fujii T, Qui D, Vincent R, Hogg JC. Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM(10)). *Am J Respir Crit Care Med*. 2001; **164**: 826-30.

35 Tan WC, Qiu D, Liam BL, Ng TP, Lee SH, van Eeden SF, D'Yachkova Y, Hogg JC. The human bone marrow response to acute air pollution caused by forest fires. *Am J Respir Crit Care Med*. 2000; **161**: 1213-7.

36 Hertel S, Viehmann A, Moebus S, Mann K, Brocker-Preuss M, Mohlenkamp S, Nonnemacher M, Erbel R, Jakobs H, Memmesheimer M, Jockel KH, Hoffmann B. Influence of short-term exposure to ultrafine and fine particles on systemic inflammation. *Eur J Epidemiol*. 2010; **25**: 581-92.

37 Peters A, Frohlich M, Doring A, Immervoll T, Wichmann HE, Hutchinson WL, Pepys MB, Koenig W. Particulate air pollution is associated with an acute phase response in men; results from the MONICA-Augsburg Study. *Eur Heart J*. 2001; **22**: 1198-204.

38 Levi M, Keller TT, van Gorp E, ten Cate H. Infection and inflammation and the coagulation system. *Cardiovasc Res*. 2003; **60**: 26-39.

39 Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med*. 2001; **164**: 1665-8.

40 Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A*. 2002; **65**: 1531-43.

41 Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation*. 2002; **105**: 411-4.

42 Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, Macnee W, Millar AM, Donaldson K, Newby DE. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *Am J Respir Crit Care Med*. 2006; **173**: 426-31.

43 Khandoga A, Stampfl A, Takenaka S, Schulz H, Radykewicz R, Kreyling W, Krombach F. Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice in vivo. *Circulation*. 2004; **109**: 1320-5.

44 Nemmar A, Hamoir J, Nemery B, Gustin P. Evaluation of particle translocation across the alveolo-capillary barrier in isolated perfused rabbit lung model. *Toxicology*. 2005; **208**: 105-13.

45 Nemmar A, Hoet PH, Dinsdale D, Vermylen J, Hoylaerts MF, Nemery B. Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. *Circulation*. 2003; **107**: 1202-8.

46 Bai N, Kido T, Suzuki H, Yang G, Kavanagh TJ, Kaufman JD, Rosenfeld ME, van Breemen C, Eeden SF. Changes in atherosclerotic plaques induced by inhalation of diesel exhaust. *Atherosclerosis*. 2011; **216**: 299-306.

47 Diez Roux AV, Auchincloss AH, Franklin TG, Raghunathan T, Barr RG, Kaufman J, Astor B, Keeler J. Long-term exposure to ambient particulate matter and prevalence of subclinical atherosclerosis in the Multi-Ethnic Study of Atherosclerosis. *Am J Epidemiol*. 2008; **167**: 667-75.

48 Hoffmann B, Moebus S, Kroger K, Stang A, Mohlenkamp S, Dragano N, Schmermund A, Memmesheimer M, Erbel R, Jockel KH. Residential exposure to urban air pollution, ankle-brachial index, and peripheral arterial disease. *Epidemiology*. 2009; **20**: 280-8.

49 Kunzli N, Jerrett M, Garcia-Esteban R, Basagana X, Beckermann B, Gilliland F, Medina M, Peters J, Hodis HN, Mack WJ. Ambient air pollution and the progression of atherosclerosis in adults. *PLoS One*. 2010; **5**: e9096.

50 Kunzli N, Jerrett M, Mack WJ, Beckerman B, LaBree L, Gilliland F, Thomas D, Peters J, Hodis HN. Ambient air pollution and atherosclerosis in Los Angeles. *Environ Health Perspect*. 2005; **113**: 201-6.

51 Quan C, Sun Q, Lippmann M, Chen LC. Comparative effects of inhaled diesel exhaust and ambient fine particles on inflammation, atherosclerosis, and vascular dysfunction. *Inhalation toxicology*. 2010; **22**: 738-53.

52 Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *Jama*. 2005; **294**: 3003-10.

53 Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, van Eeden SF. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol*. 2002; **39**: 935-42.

54 Soberanes S, Urich D, Baker CM, Burgess Z, Chiarella SE, Bell EL, Ghio AJ, De Vizcaya-Ruiz A, Liu J, Ridge KM, Kamp DW, Chandel NS, Schumacker PT, Mutlu GM, Budinger GR. Mitochondrial complex III-generated oxidants activate ASK1 and JNK to induce alveolar epithelial cell death following exposure to particulate matter air pollution. *J Biol Chem*. 2009; **284**: 2176-86.

55 Urich D, Soberanes S, Burgess Z, Chiarella SE, Ghio AJ, Ridge KM, Kamp DW, Chandel NS, Mutlu GM, Budinger GR. Proapoptotic Noxa is required for particulate matter-induced cell death and lung inflammation. *Faseb J*. 2009; **23**: 2055-64.

56 Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci (Lond)*. 2004; **107**: 343-54.

57 Hansen CS, Sheykhzade M, Moller P, Folkmann JK, Amtorp O, Jonassen T, Loft S. Diesel exhaust particles induce endothelial dysfunction in apoE-/- mice. *Toxicol Appl Pharmacol*. 2007; **219**: 24-32.

58 Tornqvist H, Mills NL, Gonzalez M, Miller MR, Robinson SD, Megson IL, Macnee W, Donaldson K, Soderberg S, Newby DE, Sandstrom T, Blomberg A. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *Am J Respir Crit Care Med*. 2007; **176**: 395-400.

59 Yamawaki H, Iwai N. Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. *Circ J*. 2006; **70**: 129-40.

60 Jacobs L, Emmerechts J, Hoylaerts MF, Mathieu C, Hoet PH, Nemery B, Nawrot TS. Traffic air pollution and oxidized LDL. *PLoS One*. 2011; **6**: e16200.

61 Lund AK, Lucero J, Harman M, Madden MC, McDonald JD, Seagrave JC, Campen MJ. The oxidized low-density lipoprotein receptor mediates vascular effects of inhaled vehicle emissions. *Am J Respir Crit Care Med*. 2011; **184**: 82-91.

62 Soares SR, Carvalho-Oliveira R, Ramos-Sanchez E, Catanozi S, da Silva LF, Mauad T, Gidlund M, Goto H, Garcia ML. Air pollution and antibodies against modified lipoproteins are associated with atherosclerosis and vascular remodeling in hyperlipemic mice. *Atherosclerosis*. 2009; **207**: 368-73.

63 Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. *Rev Esp Cardiol*. 2009; **62**: 1161-78.
64 Kita T, Kume N, Minami M, Hayashida K, Murayama T, Sano H, Moriwaki H, Kataoka H, Nishi E, Horiuchi H, Arai H, Yokode M. Role of oxidized LDL in atherosclerosis. *Ann N Y Acad Sci*. 2001; **947**: 199-205; discussion -6.
65 Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ, Nel AE. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circ Res*. 2008; **102**: 589-96.
66 Sun Q, Yue P, Kirk RI, Wang A, Moatti D, Jin X, Lu B, Schecter AD, Lippmann M, Gordon T, Chen LC, Rajagopalan S. Ambient air particulate matter exposure and tissue factor expression in atherosclerosis. *Inhalation toxicology*. 2008; **20**: 127-37.
67 Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor-and collagen-dependent thrombus formation in arterioles and venules in vivo differential sensitivity to thrombin inhibition. *Microcirculation*. 2008; **15**: 269-82.
68 Cyrys J, Stolzel M, Heinrich J, Kreyling WG, Menzel N, Wittmaack K, Tuch T, Wichmann HE. Elemental composition and sources of fine and ultrafine ambient particles in Erfurt, Germany. *The Science of the total environment*. 2003; **305**: 143-56.
69 Budinger GR, McKell JL, Urich D, Foiles N, Weiss I, Chiarella SE, Gonzalez A, Soberanes S, Ghio AJ, Nigdelioglu R, Mutlu EA, Radigan KA, Green D, Kwaan HC, Mutlu GM. Particulate matter-induced lung inflammation increases systemic levels of PAI-1 and activates coagulation through distinct mechanisms. *PLoS One*. 2011; **6**: e18525.
70 Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev*. 1999; **2**: 31-86.
71 Cassee FR, Boere AJ, Fokkens PH, Leseman DL, Sioutas C, Kooter IM, Dormans JA. Inhalation of concentrated particulate matter produces pulmonary inflammation and systemic biological effects in compromised rats. *J Toxicol Environ Health A*. 2005; **68**: 773-96.
72 Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J*. 2008; **6**: 14.
73 Tohda G, Oida K, Okada Y, Kosaka S, Okada E, Takahashi S, Ishii H, Miyamori I. Expression of thrombomodulin in atherosclerotic lesions and mitogenic activity of recombinant thrombomodulin in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1998; **18**: 1861-9.
74 Back J, Sanchez J, Elgue G, Ekdahl KN, Nilsson B. Activated human platelets induce factor XIIa-mediated contact activation. *Biochem Biophys Res Commun*. 2010; **391**: 11-7.
75 Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med*. 2005; **202**: 271-81.
76 Konings J, Govers-Riemslag JW, Philippou H, Mutch NJ, Borissoff JI, Allan P, Mohan S, Tans G, Ten Cate H, Ariens RA. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood*. 2011.
77 Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, Cassee FR, Donaldson K, Boon NA, Badimon JJ, Sandstrom T, Blomberg A, Newby DE. Diesel exhaust inhalation increases thrombus formation in man. *Eur Heart J*. 2008; **29**: 3043-51.
78 Manzanera M, Molina-Munoz ML, Gonzalez-Lopez J. Biodiesel: an alternative fuel. *Recent Pat Biotechnol*. 2008; **2**: 25-34.

Summary

Summary

Air pollution, as a part of urban life, consists of gases (carbon monoxide; CO, nitrogen oxide; NO, nitrogen dioxide; NO_2, and ozone; O_3) and small particles (particulate matter; PM) varying in aerodynamic diameter (< 10 μm; PM10, <2.5 μm; PM2.5 and < 0.1 μm; ultrafine particles; UFPs) and chemical composition. Exposure to air pollution has been linked with changes in cardiovascular health, including enhanced systemic inflammation, blood coagulation, thrombosis and atherosclerosis. Although the most responsible components of air pollution with regard to adverse health effects are not fully understood, several studies focus on PM. The effects of PM (based on their aerodynamic diameter) on blood coagulation, platelet function and thrombosis have been summarized in **chapter 1 part I**. In general, PM may enhance blood coagulation through different mechanisms and may contribute to the risk of developing cardiovascular diseases. Given the close link between inflammation and blood coagulation, PM may trigger procoagulant responses via inflammatory cytokines such as interleukin -6 (IL-6). In addition to this indirect effect of PM on blood coagulation, UFPs may directly initiate blood coagulation since direct translocation of these small particles from the lungs into circulation has been suggested by several studies. Induced platelet activation, venous and arterial thrombosis have also been associated with exposure to PM.

In addition to procoagulant and protrombotic effects, in long term, PM may also contribute to the progression of atherosclerosis via distinct pathways, which are addressed in **chapter 1 part II**. There is clear evidence from experimental and epidemiological studies that PM affect progression of atherosclerosis at different stages from the initiation phase to advanced atherosclerosis. A mechanistic outlook suggests that proapoptotic, prooxidative and inflammatory effects of PM modulate the proatherogenic events. PM induced oxidative stress and DNA damage ultimately mediates endothelial dysfunction directly (UFPs) or via systemic inflammation. Endothelial dysfunction and following accumulation of low density lipoprotein (LDL) and induced oxidation of LDL (oxLDL) are the first events related to initiation phase of atherosclerosis. At this early stage of atherosclerosis, adhesion and activation of monocytes and migration of T lymphocytes into the subendothelial space are known events. Uptake of oxLDL by macrophages leads to the formation of foam cells, a characteristic cell constituent of atherosclerotic lesions. It has been shown that PM activates leukocytes particularly monocytes and induces foam cell formation which sustains the proinflammatory responses within the vasculature. Since atherosclerosis accepted as an ongoing inflammatory disease in life time, continuous exposure to PM is leading to advance atherosclerotic plaque formation and cardiovascular complications.

In **chapter 2** of this thesis, the effects of exposure to PM10 and corresponding gas pollutants CO, NO, NO_2 and O_3 on inflammation and blood coagulation were determined in a population study with 40 healthy subjects. The time lags within 24h before blood sampling were considered to represent direct effects of air pollution whereas time lags within 24-96h before blood sampling represented indirect effects. Light-transmittance platelet aggregometry, thrombin generation (tissue factor dependent), fibrinogen, and C reactive protein (CRP) were measured in blood samples collected consecutively 13 times within a 1-year period from study subjects. Air pollution (except O3) was associated with indirect effects on platelet aggregation and thrombin generation, but not with the inflammatory markers fibrinogen and CRP.

The gaseous air pollutants, especially NO2 and CO but not PM10 are associated with direct effects on thrombin generation. Since the gas pollutants can be considered markers for motor vehicle traffic and have been shown to be highly correlated with PM2.5 and UFPs, it is mainly this subset of PM2.5 and UFPs from the overall PM air pollution that has an effect on thrombin generation.

In chapter 3, an animal exposure study is presented. In addition to human volunteer study in which the procoagulant effects of PM10 and gas pollutants are shown in **chapter 2**, PM2.5 subset of air pollution is the focus of this chapter. Fisher F344 rats, with a mild pulmonary inflammation (O_3 exposure) at the onset of exposure, were exposed for 4 weeks, 5 days/week for 6 hours a day to diluted diesel engine exhaust (PM_{DEE}), or near roadside PM (PM2.5). Changes in vascular function and tissue factor dependent blood coagulation measured by thrombin generation and tissue factor activity in the lungs were investigated in extent to pulmonary and cardiovascular effects of traffic related PM2.5 and diesel exhaust in rat. The observed changes were a reduction in white blood cell numbers, diminished levels of von Willebrand Factor (vWF) protein, and reduced lung tissue thrombin generation and tissue factor activity. Consistent with these findings, other studies also showed decreased vWF in patients with pulmonary disease and metabolic syndrome after exposure to PM. The results may be explained to either desensitization to repeated exposure or the increased expression of anticoagulant proteins (such as Thrombomodulin) as a protective mechanism against inflammation due to O_3 exposure at the onset of experiment.

Considering the small size of UFPs and their ability to translocate into the circulation, study presented in **chapter 4** is conducted. PM in different size was tested in human plasma for thrombin generation. All PM materials increased thrombin generation in human plasma via activation of factor XII (FXII) and this finding is confirmed in FXII and FXI deficient plasmas with observation of flat thrombin generation curve. PM induced thrombin generation in human plasma could be abolished by addition of corn trypsin inhibitor (CTI; a specific inhibitor for activated FXII). *In vitro* studies are also suggested that the effect of PM on activation of FXII in plasma can be modulated with both chemical composition and size of PM.

In addition to our *in vitro* findings, UFPs were intratracheally instilled in wild-type (WT) and FXII deficient ($FXII^{-/-}$) mice and plasma thrombin generation was analyzed in plasma from treated mice at 4 and 20 h post exposure. UFPs induced a transient increase in tissue factor driven thrombin formation at 4h post instillation in WT mice compared to saline instillation. Intratracheal instillation of UFPs resulted in a procoagulant response in WT mice plasma at 20 h, whereas it was entirely suppressed in $FXII^{-/-}$ mice. Based on these findings, the study is first established a role for FXII and contact activation in the sustained procoagulant response to PM shown in mice and *in vitro.* In part, this finding enlightens our understanding for PM mediated thrombus formation and clot stability.

In respect to the effects of UFPs and gas pollutants derived from diesel exhaust on tissue factor dependent thrombin generation in healthy human subjects, the study was conducted and the results are presented in **Chapter 5.** Sixteen healthy volunteers were exposed to (i) dilute diesel exhaust, (ii) pure carbon nanoparticulate, (iii) filtered diesel exhaust, or (iv) filtered air, in a

randomized double blind cross-over study. Following each exposure at 2, 6 and 24 hours, blood samples were collected and plasma thrombin generation assessed in the presence or absence of 1 pM tissue factor. In consistent with our earlier experimental study in which we demonstrated no tissue factor mediated blood coagulation in mice at 4 and 20 hrs, the study results also did not suggest any effect of the particulate or gaseous components of diesel engine exhaust on the extrinsic blood coagulation in humans. However, activation of intrinsic pathway of coagulation should be the focus of future studies in respect to prothrombotic effects of diesel exhaust.

In Chapter 6, the effects of exposure to diesel and biodiesel (an alternative to traditional petroleum diesel) particles on progression of atherosclerosis was studied. $LDL^{-/-}$ mice were intratracheally instilled to comparable concentrations of diesel and biodiesel particles for once a week, 5 times during 6 weeks and fed with high fat diet. A collar was placed on the left carotid arteries of mice on 3^{th} week. Left carotid arteries were embedded, sectioned and the atherosclerotic plaques were investigated by immunohistochemical analysis. There were no differences in plaque burden, intima/media ratio, total vessel lumen and macrophage content after both diesel and biodiesel administration compared to saline. On the other hand, exposure to biodiesel particles resulted in more vulnerable atherosclerotic plaques characterized by loss of smooth muscle cells, bigger necrotic areas, thinner fibrous cap and less collagen content than diesel particles. Additionally, atherosclerotic lesions were more proapoptotic and prooxidative after exposure to biodiesel particles compared to atherosclerotic plaques after diesel particles treatment. Although biodiesel is considered to be less hazardous, our results suggested a greater vulnerable atherosclerotic plaque formation with biodiesel particles. These effects should be considered before replacement of petroleum diesel with biodiesel.

In **chapter 7**, based on the main findings of this thesis, the effects of air pollution including PM and gas pollutants on hemostasis and atherosclerosis are discussed. Exposure to particulate air pollution contributes in the long-term to the development and progression of atherosclerosis and, in the frame of short-term exposures, triggers hypercoagulation, thrombosis. Taken together, presented studies bring a mechanistic outlook for better understanding of air pollution induced cardiovascular diseases.

Curriculum Vitae

Curriculum vitae

Evren KILINÇ was born on October 26th, 1980 in Yeşilyurt/Malatya in Turkey. He studied Physics in the faculty of science and letters in Kocaeli University, Kocaeli (Turkey) and completed his 4 years-study on July 30th, 2003. Following a post graduation programme in the Physiology department, health sciences institute, Inonu University, Malatya (Turkey), he obtained his MSc degree on July 7th, 2006 under supervision of Prof. Dr. Yunus Karakoç. While he was studying as a PhD student in the department of Biophysics, Cerrahpasa medical faculty, Istanbul University, Istanbul (Turkey), he obtained a PhD position with Prof .Dr. Hugo ten Cate. In 2007, he started to work as a PhD student at the Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands. Under supervision of Prof. Dr. Hugo ten Cate and Dr. Henri Spronk, he performed the research presented in this thesis.

LIST OF PUBLICATIONS

ORIGINAL RESEARCH ARTICLES

1. Rudez G, Janssen NAH, **Kilinc E**, Leebeek FWG, Gerlofs-Nijland ME, Spronk HMH, ten Cate H, Cassee FR, de Maat MPM.
   Effects of ambient air pollution on hemostasis and inflammation. Environ Health Perspect. 2009; 117(6): 995-1001.

2. Senen K, Topal E, **Kilinc E**, ten Cate H, Tek I, Karakoc Y, Yetkin E.
   Plasma viscosity and mean platelet volume in patients undergoing coronary angiography.
   Clinical hemorheology and Microcirculation. 2010; 44(1): 35-41.

3. Borissoff JI, Heeneman S, **Kilinç E**, Kassak P, van Oerle R, Winckers K, Govers-Riemslag J W, Hamulyak K, Hackeng TM, Daemen MJAP, ten Cate H, Spronk HMH.
   Early Atherosclerosis Exhibits an Enhanced Procoagulant State. Circulation. 2010; 122 (8): 821-830.

4. Gerlofs-Nijland E, Totlandsdal Annike I, **Kilinç E**, Boere AJF, Fokkens PHB, Leseman DL AC, Sioutas C, Schwarze PE, Spronk HM, Hadoke PWF, Miller MR, Cassee FR.
   Pulmonary and cardiovascular effects of traffic-related particulate matter: 4-week exposure of rats to roadside and diesel engine exhaust particles. Inhal Toxicol. 2010; 22(14): 1162-1173.

5. **Kilinç E**, Schulz H, Kuiper GJAJM, Spronk HMH, ten Cate H, Upadhyay S, Ganguly K, Stoeger T, Semmler-Bhenke M, Takenaka Shinji, Kreyling WG, Pitz M, Reitmeir P, Peters A, Eickelberg O, Wichmann HE.
   The procoagulant effects of fine particulate matter in vivo, Part Fibre Toxicol. 2011 Mar 15;8:12.

6. **Kilinç E**, van Oerle R, Borissoff JI, Oschatz C, Gerlofs-Nijland ME, Janssen NA, Cassee FR, Sandstrom T, Renne T, Ten Cate H, Spronk HMH.
   Factor XII Activation is Essential to Sustain the Procoagulant Effects of Particulate Matter. J Thromb Haemost. 2011; 9(7): 1359-1367.

7. **Kilinç E**, Spronk HMH, Serroyen J, Cassee FR, Mills NL, ten Cate H. Exposure to combustion derived nanoparticles does not alter tissue factor mediated thrombin generation in healthy young adults. Submitted.

8. **Kilinç E,** Borissoff JI, Haegens A, Kooter IM, Jedynska AD, Tjwa DJTH, Spronk HMH, ten Cate H. Exposure to biodiesel exhaust triggers atherosclerotic plaque destabilization through accelerated oxidant stress and apoptosis in the arterial vessel wall. Submitted.

9. **Kilinç E**, Borissoff JI, Spronk HMH, ten Cate H. Particulate matter induced atherosclerosis: a mechanistic outlook. Submitted.

NATIONAL ARTICLES

1. Rudez G, Janssen NAH, **Kilinc E**, Leebeek FWG, Gerlofs-Nijland ME, Spronk HMH, ten Cate H, Cassee FR, de Maat MPM.
   Luchtverontreiniging en hemostase. Tromnibus. 2008; 36(3): 53-56.

BOOK CHAPTER

1. **Kilinç E.**, Rudez G., Spronk H.M.H., Nemmar A., de Maat M.P.M., ten Cate H., Hoylaerts M.F., Particles, Coagulation, and Thrombosis In: Cassee FR, Mills NL, Newby DE, eds. *Cardiovascular Effects of Inhaled Ultrafine and Nano-Sized Particles*. Chicester: John Wiley and Sons Ltd, 2011.

ORAL AND POSTER PRESENTATIONS

1. **Kilinc E.**, Spronk H.M.H., ten Cate H., van Oerle R., Cassee F.R., Gerlofs-Nijland M.E., Repeated Exposure Effect of Traffic Related Air Pollutants on Thrombogenicity, 47th Annual Meeting and Toxexpo. Seattle , Washington, USA, Mar 17-20, 2008 (Poster presentation).

2. **Kilinc E.**, Kooter I.M., van Oerle R., Gerlofs-Nijland M.E., Gosens I., Cassee F.R., Spronk H.M.H., ten Cate H., Particulate matter induces a hypercoagulable state in vivo; a role for coagulation factor XII? April 8-9, 2008: Dutch Society for Thrombosis and Hemostasis (NVTH), Koudekerke, The Netherlands (oral presentation).

3. **Kilinc E.**, Spronk H.M.H., Gosens I., van Oerle R., Govers-Riemslag J.W.P., Gerlofs-Nijland M.E., Cassee F.R., ten Cate H., Activation of the intrinsic pathway of blood coagulation by particulate matter, ISTH 2009 in Boston USA (poster presentation).

4. **Kilinc E.**, Spronk H.M., van Oerle R., Gosens I., Gerlofs-Nijland M.E., Cassee F.R., Renné T., ten Cate H, The *in vivo* procoagulant effect of particulate matter is mediated through activation of factor XII, 1. Joint meeting GTH & NVTH, 24-27 February 2010 Nürnberg, Germany (oral presentation).

5. Borissoff J.I., Heeneman S., **Kilinc E.**, Kassak P., van Oerle R., Winckers K., Govers-Riemslag J., Hamulyak K., Hackeng T.M., Daemen M.J.A.P., ten Cate H., Spronk H.M.H., Localisation and activity of coagulation proteins in early human atherosclerotic plaques suggest a pro-coagulant state, 1. Joint meeting GTH & NVTH, 24-27 February 2010 Nürnberg, Germany (oral presentation).

6. Spronk H.M., **Kilinc, E.**, van Oerle, R., Hamulyak, K., Renne, T., ten Cate, H. Platelet derived microparticles induce factor XII mediated thrombin generation. 5th international conference on thrombosis and haemostasis issues in cancer, 23-25 April 2010, Bergamo Italy (poster presentation).

7. **Kilinç E**, Spronk H.M.H., van Oerle R., Oschatz C., Gosens I, Gerlofs-Nijland M.E., Cassee F.R., Renne T., ten Cate H., Activation of the intrinsic pathway of coagulation mediates the procoagulant effects of particulate matter *in vitro* and *in vivo,* NIEHS-EPA Symposium on Air Pollution and Cardiovascular Disease, 21-22 June 2010, Seattle USA (Poster presentation).

AWARDS

1. Poster prize in XXII Congress of the International Society on Thrombosis and Haemostasis. Boston, USA, July 11-16, 2009.

2. Second best oral presentation in PhD training course "Cardiac Function and Adaptation", Papendal, Arnhem, The Netherlands, Oct 12-16, 2009

Acknowledgements

Acknowledgements

There are a lot of people to thank and a lot to say but most of you know what I feel. Please accept my apologies, if I forget to mention any name. I am grateful to work with you all. It was a wonderful working environment and I have learned a lot not only about science but also about life and myself.

First, of course, Hugo ten Cate, who made this 4 years journey possible for me in the Netherlands and in lovely Maastricht. Thank you very much, Hugo, for being always kind, helpful and your great understanding. You definitely taught me how to find my way in science and sure, you will continue with many others. Henri, thank you for your patience, help and trust, we have had very hard times in the past together but we finished this nice piece at the end. Please keep being more patient for the new PhD students ;). We learn over time and step on our own feet.

I will always miss to work with you, thank you all, Karly, Arina, Rene, Patricia, Diane, Paola, Sarah, Peter, Marie Claire (MC), Rinske (Olga), Marieke, Petra, Rick, Paul, Kristien, Marina, Stella, Elisabetta. My paranymphs Joke and Geert-Jan, thank you for our great time together; you have been always good friends. Annemieke and Pieter, recently you have been part of our group and our room but I enjoyed the time with you. Julian, I will miss your friendship, there are a lot of memories, hard and fun times we have had. Thank you my friend for all. Please take care of Gergana and your daughter in Boston.

Thank you Trees, you are like my mum in the Netherlands, and always took care of me. I will miss you. Jose and Frans, I will never forget your hospitality, kindness and friendship, thanks.

Please forgive me, I never guessed this would be the hardest part of the thesis to write, I have to list all of you here and special thank personally.

Elias, Carlos, Duarte, Reyhan, Yeliz, Birol, Burak, Erkan, Ceren, Ingrid, Stefano, Deirdre, Semih, Machiel, Marion; thank you for your friendship and support.

Son olarak, Annem'e, Babam'a, Ebru, Kadir, Burcu, Ahmet ve Kardelen' e bana olan destekleri ve güvenleri için çok teşekkür ederim.